THE UNIVERSITY OF ALBERTA

ANAEROBIC BIOLOGICAL TREATMENT OF PHENOLIC WASTEWATERS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN
ENVIRONMENTAL SCIENCE

Civil Engineering

Edmonton, Alberta
Fall 1984

THE UNIVERSITY OF ALBERTA

RELEASE FORM

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TITLE OF THESIS ANAEROBIC BIOLOGICAL TREATMENT OF PHENOLIC WASTEWATERS

DEGREE FOR WHICH THESIS WAS PRESENTED DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED Fall 1984

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ANAEROBIC BIOLOGICAL TREATMENT OF PHENOLIC WASTEWATERS submitted by PHILLIP M. FEDORAK in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ENVIRONMENTAL SCIENCE.

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Dedicated to my parents

Michael G. Fedorak
September 19, 1930 - February 5, 1976

and

Margaret S. Fedorak
February 28, 1930 - January 4, 1981

ABSTRACT

The Hungate serum bottle culture method was used to assess the treatability of phenolic compounds by methanogenic consortia under batch and draw and feed conditions. Varying doses of individual compounds (phenol and 12 alkyl phenolics), synthetic mixtures of several of these, and two different industrial wastewaters were tested in batch cultures to determine their effects on the anaerobic process and fermentability. None of three sludges from anaerobic, phenolic-containing environments could degrade a larger number of phenolics than domestic sewage sludge which was routinely used as an inoculum.

Of the 13 phenolics tested, only phenol, p-cresol and m-cresol were found to be anaerobically biodegradable as demonstrated by substrate loss and corresponding methane production. Acclimation times required for phenol and p-cresol degradation ranged from 14 to 39 days. Higher concentrations required longer acclimation times. Cultures required nearly 60 days acclimation prior to m-cresol degradation. This time was shortened when other fermentable phenolics were present.

Anaerobic cultures could withstand relatively high concentrations of phenolics before methane production was inhibited. Individually, up to 1 200 mg/L phenol, 600 mg/L p-cresol, 400 mg/L m- or o-cresol, 300 mg/L of the six dimethylphenol isomers, 200 mg/L p-ethylphenol and 200 mg/L of two isomers of dihydroxytoluene were not inhibitory.

Experiments with various concentrations of either phenol or p-cresol showed that the phenolic-degrading acid-formers were inhibited at lower phenolic concentrations than were methane bacteria.

The kinetics of phenol degradation in batch cultures containing various initial phenol concentrations were studied. These data did not fit the sigmoidal substrate depletion curves predicted by the Monod model. When the initial phenol levels were between 43 and 199 mg/L, the final removal rates followed first order kinetics.

Molecular hydrogen was thought to be limiting at the time phenol degradation began. Batch cultures were then supplemented with either propionate (a hydrogen source) or H_2 at various times to enhance phenol degradation. Neither approach produced that effect. Propionate addition retarded the start of phenol degradation. This finding suggests that the phenol-degraders prefer propionate as a substrate.

A most probable number method was evaluated for the enumeration of phenol-degrading organisms. However, the method was found to be inadequate since it produced microbial numbers which were too low to account for observed phenol degradation rates.

Batch cultures receiving synthetic mixtures of fermentable and non-fermentable phenolics could selectively degrade the former compounds if the total phenolic concentration was near or below 700 mg/L. The treatability of wastewaters from a coking process and a coal conversion (H-coal) process containing total phenolics of 410 and 7 600 mg/L, respectively was also tested. In both cases, fermentable phenolics accounted for >85% of the phenolics. The coke effluent contained a non-ether-extractable component (presumably cyanide) which inhibited the anaerobic process at concentrations >30% (V/V). Concentrations of >8% H-coal effluent were inhibitory and this effect was attributed to non-phenolic, ether-extractable components. At concentrations greater than 10% to 12%, phenolics themselves would be inhibitory.

Semicontinuous cultures were maintained on feeds of 2% and 4% V/V H-coal effluent for stable periods ranging from 17 to 53 days. The length of the stable period decreased with increasing substrate mass loading rate. Analysis of the dynamic response of the reactors at the end of the stable period indicated a sudden and large decrease in m-cresol removal capacity for all reactors. Reactors receiving higher substrate mass loadings later exhibited similar behavior for p-cresol and ultimately phenol in the case of the highest loading.

ACKNOWLEDGEMENTS

Financial support for much of this investigation was provided by the Alberta/Canada Energy Resources Research Fund administered by the Department of Energy and Natural Resources of the Province of Alberta. The support through this fund of the activities of the Hydrocarbon Research Centre of the University of Alberta provided additional funding for this project. Grants from the Alberta Environmental Research Trust Fund and the Natural Sciences and Engineering Council of Canada are also gratefully acknowledged. A portion of this study was undertaken as part of a subcontract with Dearborn Environmental Consulting Services on behalf of the Wastewater Technology Centre, Environmental Protection Service, Environment Canada.

I thank Val Williams, Shauna Mercer and Barb Thomson for their fine technical assistance.

I am indebted to Dr. D.W.S. Westlake of the Department of Microbiology for his help throughout this project. I also acknowledge the assistance of Dr. D.R. Boone who introduced me to the use of the Hungate serum bottle method.

I also thank my adviser, Dr. Steve E. Hrudey, for his guidance, his critical review of the experimental results, and his resourcefulness in obtaining funding for this project.

Finally, I thank my loving wife, Jeanette, for her patience and understanding over the course of this post-graduate program.

TABLE OF CONTENTS

Chapter Page

ABSTRACT v

ACKNOWLEDGEMENTS viii

LIST OF TABLES xiv

LIST OF FIGURES xvi

LIST OF ABBREVIATIONS xxi

1. INTRODUCTION 1

2. LITERATURE REVIEW 4

2.1 Wastewaters Containing Phenolics 4

2.2 Laboratory Methods Used To Study The Methanogenic Fermentation of Phenolics 9

2.2.1 Anaerobic Methods 11

2.2.2 Types of Reactors Used for Laboratory Studies of Methanogenic Fermentation 14

2.3 Anaerobic Microbial Metabolism of Aromatic Compounds 18

2.3.1 Studies Using Pure Substrates 19

2.3.2 Anaerobic Treatment of Phenolic Wastewaters 38

2.4 Analytical Methods 43

2.4.1 Analysis of Phenolics 43

2.4.2 Methane Analysis 45

3. MATERIALS AND METHODS 47

3.1 Anaerobic Batch Culture Methods 47

3.2 Analytical Methods 51

3.2.1 Substrate Analyses 51

3.2.2 Methane Analysis 52

3.2.3 Gas Volume Measurements 54

3.2.[illegible]1 Description of Gas Volume Measuring Apparatus 54

3.2.3.2 Use of the Apparatus 56

3.2.4 Statistical Methods 59

4. EVALUATION OF ANALYTICAL METHODS 61

4.1 Substrate Analyses 61

4.2 Methane Analyses 64

4.3 Gas Volume Measurements 67

5. FERMENTABILITY AND EFFECTS OF INDIVIDUAL PHENOLICS IN BATCH CULTURES 75

5.1 Procedures 76

5.1.1 Initial Screening Experiments 76

5.1.2 Cultures Supplemented With Fermentable Substrates 77

5.1.3 Substrate Loss From Methane Producing Cultures 78

5.1.4 Fermentable Substrate Concentration and Acclimation Time 79

5.1.5 Ultimate Gas Production from Phenol and p-Cresol 79

5.1.6 The Ability of Alternate Sources of Inocula to Degrade Phenolics 80

5.2 RESULTS AND DISCUSSION 83

5.2.1 Initial Screening Experiments 83

5.2.2 Cultures Supplemented With Fermentable Substrates 87

5.2.3 Substrate Loss From Methane Producing Cultures 103

5.2.4 Fermentable Substrate Concentration and Acclimation Time 107

5.2.5 Ultimate Gas Production From Phenol and p-Cresol 110

5.2.6 The Ability of Alternate Sources of Inocula to Degrade Phenolics 117

6. FERMENTATION OF PHENOL IN BATCH CULTURES 123

6.1 Procedures 124

6.1.1 Kinetics of Phenol Degradation in Batch Cultures 124

6.1.2 Propionate-supplemented Batch Cultures ..125

6.1.3 Hydrogen-supplemented Batch Cultures 127

6.1.4 MPN Method 128

6.2 Results and Discussion 129

6.2.1 Kinetics of Phenol Degradation in Batch Cultures 129

6.2.2 Propionate-supplemented batch cultures ..138

6.2.3 Hydrogen-supplemented Batch Cultures 147

6.2.4 MPN Results 151

7. FERMENTATION OF SYNTHETIC MIXTURES OF PHENOLICS IN BATCH CULTURES 166

7.1 Procedures 167

7.1.1 Fermentability of Phenol and p-Cresol in Mixtures of Non-fermentable Phenolics ...167

7.1.2 Fermentability of m-Cresol Alone and in Mixtures of Phenolics 169

7.2 Results and Discussion 171

7.2.1 Fermentability of Phenol and p-Cresol in Mixtures of Non-fermentable Phenolics ...171

7.2.2 Fermentability of m-Cresol Alone and in Mixtures of Phenolics 179

8. BATCH CULTURE FERMENTATIONS OF PHENOLIC WASTEWATERS 189

8.1 Procedures 191

8.1.1 Analyses of the Two Industrial Wastewaters 191

8.1.1.1 Major Phenolic Compounds 191

8.1.1.2 Volatile Organic Acids 192

8.1.2 Batch Cultures Receiving Unextracted Wastewaters 193

8.1.2.1 Coke Effluent 193

8.1.2.2 H-coal Effluent 194

8.1.3 Batch Cultures Receiving Ether-extracted Wastewaters 195

8.1.3.1 Extracted Coke Effluent 195

8.1.3.2 Extracted H-coal Effluent 195

8.1.4 Cultures Receiving Reconstituted H-coal Effluent 196

8.1.4.1 Preparation of Reconstituted H-coal Effluent 196

8.1.4.2 Batch Cultures with Reconstituted H-coal Effluent 197

8.1.5 Ultimate Methane Production from H-coal Effluent 198

8.2 Results and Discussion 198

8.2.1 Chemical Characteristics of the Industrial Wastewaters 198

8.2.2 Batch Cultures Receiving Unextracted Wastewaters 202

8.2.2.1 Coke Effluent 202

8.2.2.2 H-coal Effluent 206

8.2.3 Batch Cultures Receiving Ether-extracted Wastewaters 212

8.2.3.1 Extracted Coke Effluent 214

8.2.3.2 Extracted H-coal Effluent 219

8.2.4 Cultures Receiving Reconstituted H-coal Effluent 222

8.2.5 Ultimate Methane Production from H-coal Effluent 229

9. FERMENTATION OF PHENOLICS IN SEMICONTINUOUS CULTURES 232

9.1 Procedures 232

9.1.1 Culture Methods 232

9.1.2 Feed Solution Composition 235

9.1.3 Cultures Receiving Pure Phenolic Substrates 236

9.1.4 Cultures Receiving Diluted H-coal Effluent 237

9.2 Results and Discussion 237

9.2.1 Cultures Receiving Pure Phenolic Substrates 240

9.2.2 Cultures Receiving Diluted H-coal Effluent 246

10. SUMMARY AND CONCLUSIONS 277

REFERENCES 285

LIST OF TABLES

Table	Description	Page
2.1	Industrial sources and concentrations of phenol	6
2.2	Phenol and alkyl phenolics in various industrial wastewater streams	8
2.3	Selected studies on anaerobic digestion using various laboratory-scale reactors	15
2.4	Anaerobic metabolism of aromatic compounds	22
3.1	Stock solutions used in growth medium	48
3.2	Composition of growth medium	49
4.1	Comparison of gas volume measurements made with and without the insulating sample bottle holder	69
4.2	Quantitative measurement of a known amount of methane in sealed serum bottles	72
5.1	Substrate concentrations used in fermentability studies with various inocula from phenolic-containing environments	81
5.2	Summary of initial screening experiments	85
5.3	Effects of some alkyl phenolics on the conversion of VOAs to methane	88
5.4	Effects of fermentable phenolic substrate concentration on acclimation time	108
5.5	Methane and total gas production from phenol and p-cresol	113
6.1	Summary of phenol removal rates and volatile nonfiltrable residue concentrations in batch cultures containing various amounts of phenol	134
6.2	Survey of applications of the MPN technique	153
6.3	Summary of MPN results from attempts to enumerate PDU in anaerobic sludges	157
6.4	Literature values for parameters required to consider the validity of the PDU MPN values	159
7.1	Concentrations of phenolics in batch cultures receiving mixtures of these compounds	168

Table	Description	Page
7.2	Phenolics added to batch cultures to test the fermentability of m-cresol	170
8.1	Analyses of phenolic wastewaters received from Dearborn Environmental Consulting Services	199
8.2	Concentrations of various phenolics in the two industrial wastewaters used in batch culture studies	201
9.1	Summary of semicontinuous cultures maintained on diluted H-coal effluent	238
9.2	Removal of m/p-cresol from semicontinuous cultures receiving diluted H-coal effluent	255
9.3	Concentrations of m- and p-cresol in composite effluent samples from semicontinuous cultures receiving diluted H-coal effluent	257
9.4	Estimated first order constants for m-cresol removal in semicontinuous cultures receiving diluted H-coal effluent	268
9.5	Summary of methane production rates in semicontinuous cultures receiving diluted H-coal effluent	274

LIST OF FIGURES

Figure Page

2.1 Comparison of some intermediates formed during anaerobic and aerobic microbial degradation of benzoic acid....................................21

3.1 Apparatus for measuring the volume of gas produced by methanogenic cultures in serum bottles...55

4.1 Typical standard curves for phenol analysis.......62

4.2 Comparison of the analyses of laboratory prepared methane standards with those of commercially prepared "certified" methane standards.......................................65

4.3 Measured volumes of air displaced by known amounts of glycerol added to sealed serum bottles.70

5.1 Methane production in batch cultures containing various concentrations of 2,3-dimethylphenol - with VOA supplementation...........................90

5.2 Microbial activities leading to methane production in the presence of fermentable phenolics.......................................92

5.3 Methane production in batch cultures containing various concentrations of phenol - without VOA supplementation................................93

5.4 Methane production in batch cultures containing various concentrations of phenol - with VOA supplementation................................95

5.5 Methane production in batch cultures containing various concentrations of p-cresol - without VOA supplementation................................97

5.6 Methane production in batch cultures containing various concentrations of p-cresol - with VOA supplementation................................98

5.7 Methane production in batch cultures containing various concentrations of phenol - supplemented with nutrient broth and glucose (NBG)...........101

Figure Page

5.8 Methane production in batch cultures containing various concentrations of p-cresol - supplemented with nutrient broth and glucose (NBG).....102

5.9 Substrate loss and methane production in a batch culture initially containing 450 mg/L phenol.....104

5.10 Substrate loss and methane production in a batch culture initially containing 160 mg/L p-cresol...105

5.11 Ultimate quantity of methane produced from phenol in batch cultures.....112

5.12 Ultimate quantity of methane produced from p-cresol in batch cultures.....115

6.1 Selected substrate depletion curves from phenol-containing batch cultures.....131

6.2 Rate of phenol removal as a function of initial substrate concentration.....135

6.3 Degradative pathway for phenol in a methanogenic consortium.....139

6.4 Substrate depletion from phenol-containing batch cultures supplemented with sodium propionate at various times.....141

6.5 Methane production in batch cultures supplemented with sodium propionate at time zero.143

6.6 Methane production in batch cultures supplemented with sodium propionate on day 15....145

6.7 Substrate depletion from phenol-containing batch cultures supplemented with H_2 at various times...149

7.1 Substrate loss and methane production in batch culture F (trial 1) containing phenol and p-cresol.....173

7.2 Substrate loss and methane production in batch culture B (trial 1) containing a mixture of six phenolics with a total concentration near 730 mg/L.....175

Figure Page

7.3 Phenolic analyses of culture B (trial 1) at the time of inoculation and after 49 days incubation.176

7.4 Substrate loss and methane production in batch culture containing a mixture of six phenolics with a total concentration near 372 mg/L.........178

7.5 Substrate loss and methane production in batch cultures receiving individual phenolics..........180

7.6 Substrate loss from batch cultures receiving m-cresol mixed with other phenolics..............183

7.7 Reduction of the acclimation time for m-cresol degradation when other phenolics were present in batch cultures....................................184

7.8 Typical methane production curves from batch cultures receiving m-cresol mixed with other fermentable phenolics............................187

8.1 Methane production in batch cultures containing various concentrations of coke effluent - without VOA supplementation.........................203

8.2 Methane production in batch cultures containing various concentrations of coke effluent - with VOA supplementation...............................205

8.3 Methane production in batch cultures containing various concentrations of H-coal effluent - without VOA supplementation.......................207

8.4 Substrate analyses of batch cultures containing H-coal effluent at various times.................209

8.5 Methane production in batch cultures containing various concentrations of H-coal effluent - with VOA supplementation..............................213

8.6 Methane production in batch cultures containing various concentrations of extracted coke effluent - without VOA supplementation...........215

8.7 Methane production in batch cultures containing various concentrations of extracted coke effluent - with VOA supplementation..............217

Figure Page

8.8 Methane production in batch cultures containing various concentrations of ether extracted H-coal effluent....221

8.9 Methane production in batch cultures containing either H-coal effluent or reconstituted H-coal effluent....225

8.10 Methane production in batch cultures containing various concentrations of reconstituted H-coal effluent....226

9.1 Procedures used to maintain draw and feed cultures....234

9.2 Methane production from phenol degradation by a semicontinuous culture. Experimental data compared to predicted values....242

9.3 Methane production from p-cresol degradation by a semicontinuous culture. Experimental data compared to predicted values....245

9.4 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 2 mL of 2% H-coal effluent each day....248

9.5 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 3 mL of 2% H-coal effluent each day....249

9.6 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 4 mL of 2% H-coal effluent each day....250

9.7 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 2 mL of 4% H-coal effluent each day....251

9.8 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 3 mL of 4% H-coal effluent each day....252

9.9 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 4 mL of 4% H-coal effluent each day....253

Figure Page

9.10 Comparison of observed m-cresol concentrations in the effluent from culture A to those expected under washout conditions.........................259

9.11 Comparison of observed m-cresol concentrations in the effluent from culture B to those expected under washout conditions.........................260

9.12 Comparison of observed m-cresol concentrations in the effluent from culture C to those expected under washout conditions.........................261

9.13 Comparison of observed m-cresol concentrations in the effluent from culture D to those expected under washout conditions.........................262

9.14 Comparison of observed m/p-cresol concentrations in the effluent from culture E to those expected under washout conditions for both isomers........263

9.15 Comparison of observed m/p-cresol concentrations in the effluent from culture F to those expected under washout conditions for both isomers........264

LIST OF ABBREVIATIONS

BOD	biochemical oxygen demand
DOC	dissolved organic carbon
GC	gas chromatography
MPN	most probable number
NBG	nutrient broth and glucose supplement
STP	Standard temperature and pressure conditions (273° K and 1-atmosphere pressure)
PDU	phenol-degrading unit
TOC	total organic carbon
U	statistically uniform labelling in a ^{14}C compound
VOA	volatile organic acids
Vol	volume
θ	hydraulic retention time

1. INTRODUCTION

Two problems of current concern to industrialized nations are the availability of energy resources and environmental pollution. Methanogenic fermentation of phenolics is related to both of these problems in several ways. Phenolics are toxic pollutants which can cause taste and odour problems in fish and drinking water at very low concentrations. Therefore, receiving waters must be protected from these pollutants. Phenolics are common contaminants in the wastewaters of hydrocarbon processing industries including petroleum refineries. As the supply of conventional crude oil contracts, new sources of energy are being sought. These include oil sands and shale oils as well as synthetic fuels produced by coal conversion (liquefaction and gasification) processes. Such conversion methods have been found to produce large amounts of phenolic wastewaters which must be treated at reasonable cost. The common aerobic biological methods of treatment require large inputs of energy to supply oxygen for the microorganisms which are able to degrade these compounds. In addition, aerobic processes generate more biomass, in the form of sludge, than anaerobic processes. Biological sludges require treatment and disposal. Overall, the anaerobic process has a lower energy demand making it an attractive alternative. Thus, the anaerobic process may be an energy saving means of treating wastewaters from energy producing industries.

The anaerobic process yields methane. In recent years there has been a considerable amount of research activity concerning energy production from renewable resources. Much of this interest is directed towards production of "biogas", which is methane derived from the anaerobic degradation of materials such as straw, wood chips, municipal refuse, etc. In the case of wood as a source of biogas, much of the chemical energy therein is found as lignin. Upon alkaline hydrolysis, this polymer gives a variety of phenolic compounds that may serve as substrates for the methanogenic fermentation.

The types of phenolics present in wastewaters from petroleum and coal conversion industries are quite different from those resulting from lignin decomposition. The former group contain mainly alkyl side chains while the latter group is more highly oxygenated and contain carboxy and methoxy substituents.

The current technical literature provides data on the susceptibilities of various phenolics toward anaerobic degradation and some information on laboratory studies of the treatment of phenolic wastewaters by methanogenic cultures. Most of these studies report on the success or failure of the method with relatively little data on the effects that these toxic substrates have on the cultures. Phenolics reported as nondegradable by some workers have been found to be fermented to methane by others. Thus, there is some doubt as to which compounds are degradable by anaerobic methods.

The major objectives of this study were:

1. To examine and reevaluate the degradability of a variety of alkyl phenolics under anaerobic batch culture conditions.
2. To determine the effects of phenolic concentrations on the microbial activities in methanogenic consortia.
3. To measure the ultimate absolute methane yield per unit mass of phenol and p-cresol in batch cultures.
4. To evaluate the removal of fermentable phenolics from synthetic mixtures of phenolics and from industrial wastewaters in batch methanogenic cultures.
5. To establish, maintain and evaluate the removal of phenolics and subsequent methane production of semicontinuous (draw and feed) cultures growing on pure phenolics or industrial wastewaters.

Peripheral investigations considered (a) the kinetics of phenol degradation in batch cultures, (b) the effects of hydrogen and a hydrogen donor (propionate) on the degradation rate of phenol, and (c) the use of a MPN techique for the enumeration of phenol-degrading units in anaerobic sludge. These studies were intended to provide a better basic understanding of the microbiology and biochemistry of anaerobic phenol degradation.

2. LITERATURE REVIEW

Literature on four major topics has been surveyed and relevant references have been discussed in this section. These topics include an overview of the industrial sources of wastewaters which contain significant amounts of phenolics with special attention given to those reports which indicate the presence of specific alkyl phenols in the wastewater. The general laboratory methods used to work with anaerobic cultures and those used to study anaerobic digestion with various reactor types are reviewed. Applications of these anaerobic methods to the study of degradation of pure phenolic substrates and to the laboratory-scale treatment of complex phenolic wastewaters have been surveyed. Finally, a review of the analytical methods required to monitor the activities of anaerobic cultures being fed phenolics is presented.

2.1 Wastewaters Containing Phenolics

The occurrence and approximate overall quantity of phenolic compounds are commonly determined by the non-specific 4-aminoantipyrine colourimetric method (APHA, 1980). More recently, phenolics in wastewaters have been analyzed using gas chromatography (GC), GC-mass spectrometry and high performance liquid chromatography (HPLC) giving a better indication of which compounds are present. If all of the compounds in the sample are sufficiently well resolved by the chromatographic system, it is possible to identify and quantitate each phenolic. However, few extensive analyses of this type are available.

In general, phenolic wastewaters contain phenol as the major compound of this group with smaller quantities of derivatives of phenol. Babich and Davis (1981) have reviewed the environmental and health risks of phenol along with many of the industrial wastewater sources of this contaminant. Patterson (1975) has provided a list of industries which produce phenolic wastes and gives typical concentration ranges of phenol in these wastewaters. Table 2.1 summarizes some of these sources and shows that a number of industries produce a wide range of phenol concentrations in their wastewaters. Khan et al. (1982) found phenol at 420 000 mg/L in an aircraft paint stripping bath. Akhtar et al. (1978) reported phenol at 250 mg/L in SYNTHOIL reactor scrub water when distilled water was used for the scrub while 5 100 mg/L phenol was observed when a caustic solution was used.

The concentrations of phenolics (or phenols) have often been reported as a general class. For example, Luthy et al. (1977) gives values found in a number of wastewater streams in pilot scale coal gasification plants. These range from less than 1 mg/L for ash slurry water to 6 000 mg/L in the product gas quench condensate. Similarly, Bhattacharyya and Middleton (1980) have reported phenols at 1 400 mg/L in raw ammoniacal liquor from a coke plant while the combined wastewater from that plant contained 130 mg/L phenols.

Table 2.1 Industrial sources and concentrations of phenol. After Patterson (1975).

Industry	Concentration (mg/L)
Coking plant	
Weak ammonia liquor, without dephenolization	580 - 10 000
Weak ammonia liquor after dephenolization	4 - 332
Wash oil still wastes	30 - 150
Oil Refineries	
Sour water	80 - 185
General wastewater	10 - 100
API separator effluent	0.3 - 6.8
Petrochemical	
Benzene refinery	210
Tar distillation	300
Nitrogen works	250
Orlon manufacturing	100 - 150
Plastics factory	600 - 2 000
Phenolic resin production	1 600
Fiberboard factory	150
Fiberglass manufacturing	40 - 400
Aircraft maintenance	200 - 400

Buikema et al. (1979) presented an extensive literature survey on the sources of numerous synthetic phenolics (e.g. pesticides) and naturally occurring phenolics. They also list the phenolics found in a variety of industrial wastewaters. Table 2.2 provides a summary sample of some of the individual and isomeric groups of alkyl phenolics recently reported for industrial wastewaters.

Another source of phenolic wastewaters is the pulp and paper industry. Chernousov et al. (1972) found mainly guaiacol (o-methoxyphenol) (41.8%) with smaller quantities of phenol, o-cresol, 2,5-xylenol (2,5-dimethylphenol), catechol (1,2-dihydroxybenzene) and 4-methylcatechol in the ether-soluble part of black liquor. The organic matter in sulfate liquor contained approximately 5% phenols. Of the phenolics found, guaiacol comprised 55 to 60% (Chernousov et al., 1975). McKague (1981) also found guaiacol as the predominant phenolic, with vanillin, 4-hydroxy-3-methoxyacetophenone and others present in unbleached whitewater. He also found a variety of chlorinated phenolics including 3,4,5-trichloroguaiacol and 4,5-dichloroguaiacol in bleach plant wastewaters. Although many other sources have reported the occurrence of specific phenolics in industrial wastewater, these examples serve to illustrate their importance as industrial pollutants.

Table 2.2 Sample of phenol and alkyl phenolics in various industrial wastewater streams.

Reference	Industry	Type of Sample	Compounds and concentrations (mg/L unless otherwise specified)
Baird et al. (1976)	Petroleum refinery	final effluent	phenol (0.88); m-cresol (0.75).
Baird et al. (1976)	Petroleum refinery	discharge to sewer	phenol (3 016); o-cresol (5 842) m- and p-cresol (56); 2,3-DMP[1] (8 177); 2,4- and 2,5-DMP (963); 2,6-DMP(1 547).
Ho et al. (1976)	Oil shale retorting	by-product water	phenol(10); o-cresol(30); m- and p-cresol(20).
Ho et al. (1976)	Synthane coal gasification	by-product water	phenol(2 100); o-cresol(670); m- and p-cresol(1 800); 2,6-DMP(40); 2,5-DMP(250); 3,5-DMP(230); 2,3-DMP(30); 3,4-DMP(100); o-ethylphenol(30), 1-naphthol(10); 2-naphthol(30).
Neufeld and Spinola (1978)	Coal gasification	process wastewater	phenol and o-cresol (2 209); m- and p-cresol and 2,4- and 2,5-DMP(1 626); 2,6-DMP (24); 2,3-DMP (50); 3,5-DMP and m- and p-ethylphenol (366); 3,4-DMP (119); 3-ethyl-5-methylphenol (66); C_3-phenol (40).
Pellizzari et al. (1979)	In Situ coal gasification	product water	phenol (270); p-cresol (10.2); DMP isomers (10); C_3-phenols (1.2).
Pellizzari et al. (1979)	In situ coal gasification	process water	phenol (50 000); o-cresol (8 000); DMP isomers (4 265); C_3-phenols (1 395); ethylphenols (855).
Pellizzari et al. (1979)	Low-BTU gasification	aqueous condensate	phenol (0.28); o-cresol (4.8); m-cresol (4.5); p-cresol (0.04); DMP isomers (7.4); C_3-phenols (0.1); ethylphenols (1.4).
Barbour and Guffey (1981)	Experimental tar sands recovered by in situ burning	water from recovery separator	phenol (9.0% of alkaline extract); o-cresol (2.4%); m-cresol (3.1%); p-cresol (0.9%). Also present were isomers of DMP, C_3-phenols.
Gebhart et al. (1981)	In situ coal gasification	aqueous effluents	phenolics detected: phenol, cresols, DMP's C_3-phenols, C_4-phenols.
Dark (1981)	Coal gasification	aqueous effluents	phenolics detected: phenol, cresols.

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[1] DMP = dimethylphenol.

2.2 Laboratory Methods Used To Study The Methanogenic Fermentation of Phenolics

Anaerobic digestion is a microbiological process in which organic matter is converted to methane and carbon dioxide. The overall conversion has been considered to involve two distinct groups of bacteria (Toerien and Hattingh, 1969; Kirsch and Sykes, 1971; Hobson, 1973). These are the non-methanogens, which hydrolyze and ferment complex materials to volatile organic acids (VOA) (mainly acetic and formic) and the methanogens which convert acetate, formate, and H_2/CO_2 to methane. More recently, four different trophic groups of bacteria have been recognized within the anaerobic digestion process (Zeikus, 1979 and 1982). These include:

1. the hydrolytic bacteria which ferment a wide variety of complex organics (i.e. polysaccharides, lipids and proteins) to a variety of end products such as acetic acid, H_2/CO_2, one carbon (unicarbon) compounds, organic acids larger than acetic acid, and neutral compounds larger than methanol.
2. the hydrogen producing acetogenic bacteria which can ferment organic acids larger than acetic acid (e.g. propionate, butyrate) and neutral compounds larger than methanol to acetate and H_2.
3. the homoacetogenic bacteria which can catabolize unicarbon compounds (e.g. H_2/CO_2 or HCOOH) or hydrolyze multicarbon compounds to produce acetate.

4. the methanogenic bacteria that produce methane from H_2/CO_2, methanol, formic acid, methylamine, CO, and acetic acid.

The first three trophic groups are considered to be non-methanogens or acid-formers and consist of both strict and facultative anaerobes. However, Toerien et al. (1967) and Mah and Sussman (1968) have shown that the strict anaerobes are the more numerous.

The methane-producing bacteria are among the strictest anaerobes known. They are killed more rapidly by relatively short exposures to air than other anaerobic forms (Bryant, 1974). Smith and Hungate (1958) showed that as little as 0.03% oxygen in the gas phase completely inhibited the growth of *Methanobacterium ruminantium* in pure culture.

In order to carry out complete anaerobic digestion of a fermentable substrate, balanced growth of both the non-methanogens and the methanogens must be maintained. Since the methane bacteria are the more fastidious group in terms of favourable growth ranges for temperature, pH, redox potential and oxygen concentration, the environmental or culture conditions must be suitable for the metabolic activities of these slow-growing organisms. Otherwise, complete digestion of the substrate cannot be assured. Thus, experimental growth conditions for laboratory studies of anaerobic digestion are established to cater to the methanogenic bacteria.

2.2.1 Anaerobic Methods

Although the ability of microorganisms to grow in the absence of oxygen was first described in 1861 by Pasteur (Sonnenwirth, 1972) present knowledge of the metabolism of strict anaerobes lags far behind that of aerobes. This arises from the difficulty in culturing these fastidious organisms. Hungate (1950) introduced the roll tube technique for studying strict anaerobes from the rumen. This method has been improved and described elsewhere (Hungate, 1969; Latham and Sharpe, 1971; Holdeman and Moore, 1972).

The strict anaerobic technique requires that the inoculum never be exposed to air during the transfer procedure and that the medium is prereduced at the time of inoculation. Oxygen-free gases are used to:

a. displace oxygen from the medium,
b. occupy the headspace of the culture tubes and
c. flush all pipettes and syringes used to transfer medium or cultures.

Oxygen is removed by passage of the gases through a Pyrex glass column packed with copper ribbon heated to approximately 350°C (Hungate 1969).

Oxygen is removed from the medium by sparging with O_2-free gas and/or boiling. Aliquots are then dispensed into tubes or bottles which have been flushed with O_2-free gas. These are sealed with butyl rubber stoppers (which are impermeable to oxygen) and autoclaved. Prior to inoculation, a reducing agent such as sodium thioglycolate, cysteine, sodium sulfide, hydrogen sulfide or dithionite is added to lower the redox potential to a level suitable for growth of the anaerobes. In most cases, the medium contains a redox indicator such as resazurin which turns colorless when it is reduced. Inoculation and culture transfers can be done at a laboratory bench with facilities to flush syringes and the headspaces of sample bottles and culture tubes with O_2-free gases. Alternatively, an anaerobic glove box can be used (Aranki and Freter, 1972).

The roll tube method has been used for the enumeration of methanogens (Siebert et al., 1968) and non-methanogens (Toerien et al., 1967 and 1968) in anaerobic digesters. The method also allows the isolation and characterization of methanogens (Smith and Hungate, 1958; Paynter and Hungate 1968) and non-methanogens (Toerien et al., 1968; Toerien 1970). It is generally used to obtain and maintain pure cultures rather than to study mixed culture phenomena such as methane production from complex substrates. However, it has been used to study mixtures of pure cultures, known as cocultures, to determine the interaction between species (Boone and Bryant, 1980).

A more common technique used for the study of methane production from various substrates is the serum bottle modification of the Hungate method proposed by Miller and Wolin (1974). This method usually uses a prereduced liquid medium in a serum bottle which is sealed with a butyl rubber stopper. The inoculum and substrates can be easily added by means of a syringe which has been flushed with O_2-free gas. Similarly, aliquots of the liquid medium can be removed for residual substrate analysis or as a source of inoculum for subsequent cultures. As well, gas samples can be withdrawn for compositional analysis. This method has recently been used by Owen _et_ _al_. (1979) to test both the biodegradability and the toxicity of potential feed sources for anaerobic digestion. In the latter case, acetate and propionate, which are readily fermented to methane, are added to the medium to determine whether the methanogens are susceptible to toxic effects of the feed.

It should be noted that when inocula are derived from methanogenic environments such as anaerobic digesters, rumen fluid or fermenting muds, the "strictness" of the anaerobic method can be relaxed somewhat and methane producing cultures will survive. That is, if small amounts of oxygen are present or the redox potential of the medium is too high for growth of the methanogens, the facultative anaerobes quickly reduce the medium to a redox potential suitable for methane fermentation. During this adaptation period, the methanogens are thought to persist in anaerobic microenvironments within the inoculum where they are protected from short-term unfavorable redox conditions.

2.2.2 Types of Reactors Used for Laboratory Studies of Methanogenic Fermentation

There are basically two culture methods which are used to study the anaerobic fermentation of various substrates. The batch culture, in which the substrate is added only once to the culture, and the continuous or semicontinuous culture in which fresh substrate is added either constantly or periodically.

Each reactor system requires a method for maintaining a constant temperature or for monitoring temperature, as well as a gas collecting and sampling system. The continuous or semicontinuous systems require ports to allow feeding and withdrawal of effluent without losing or adding gas. Often the reactors have mixing devices to maintain good contact between the microorganisms and the substrate. Hawkes and Young (1980) have recently reviewed the design and operation of laboratory-scale digesters.

Many workers have used a variety of reactor designs to study the anaerobic digestion of different compounds and wastewaters. Table 2.3 is a summary of some of the more recent reports with special reference to those studies involving phenolics or phenolic wastewaters. As indicated by the relative number of entries in Table 2.3, the batch method and the single stage reactor are the most commonly used because of their simplicity.

Methods which allow the mean cell residence time to be greater than the hydraulic retention time (i.e. systems with

Table 2.3 Selected studies on anaerobic digestion using various laboratory-scale reactors.

Reactor Type	Reference	Phenolics specifically used as substrate	Comments
Batch	Chmielowski *et al.* (1965)	Yes	Seventeen different phenolics tested. Each batch culture was fed increasing amounts of substrate.
	Sykes and Kirsch (1972)	No	Tested the inhibitory effects of carbon tetrachloride on anaerobic digestion.
	Chou *et al.* (1978)	Yes	Fifty-three substrates including 9 aromatic compounds tested for methane fermentation using Hungate serum bottle method and Warburg respirometer.
	Healy and Young (1978)	Yes	Phenol and catechol degradation tested using Hungate serum bottle method.
	Healy and Young (1979)	Yes	Anaerobic degradation of 11 aromatic compounds tested by Hungate serum bottle method.
	Yang *et al.* (1980)	No	Tested the inhibitory effects of cyanide and chloroform on anaerobic digestion.
	Balba and Evans (1980b)	Yes	Determined the intermediates in the anaerobic metabolism of 3 aromatic amino acids.
	Karube *et al.* (1980)	No	Immobilized cells tested for the ability to ferment an alcohol factory's wastewater.
	Bouwer *et al.* (1981)	No	Serum bottle method used to study the degradation of halogenated methanes and ethanes.
Single Stage	Chmielowski and Wasilewski (1966)	Yes	Draw and feed methods used to study the dynamics of phenol, p-cresol and resorcinol degradation.
	Sykes and Kirsch (1972)	No	Draw and feed cultures used to study the inhibitory effects of carbon tetrachloride.
	Ossio and Fox (1980)	No	After ammonia removal and nutrient addition, wastewater from an oil shale retorting process was tested with up to 90% BOD removal.

Table 2.3 (cont.)

Reactor Type	Reference	Phenolics specifically used as substrate	Comments
	Hawkes and Young (1980)	No	Discuss design and operation of lab-scale single stage digesters. Poultry litter treated in their digester.
	Winter and Cooney (1980)	No	Fermentation of cellulose and short chain fatty acids.
	Lane (1980)	No	Studied the formation of aromatic acids during the digestion of citrus peel. Used small scale (150 cm^3) and large scale (10 dm^3 and 4540 dm^3) digesters.
	Neufeld *et al.* (1980)	Yes	Draw and feed 10-L reactors used to study kinetics of phenol degradation under non-methane producing conditions.
	Balba *et al.* (1981)	Yes	After initial batch enrichment, cultures were grown under continuous-feed conditions. The intermediates in p-cresol degradation were determined.
	Dague (1981)	Yes	Wastewaters, high in phenols, from pyrolysis processes were mixed with domestic primary sludge and tested for toxicity and treatability.
Two Stage with sludge return	Massey and Pohland (1978)	No	Used two such reactors in series to study kinetic controls for phase separation method.
	Anderson *et al.* (1980)	No	High-strength wastewaters from various food industries treated in 25-L completely mixed reactor.
Anaerobic filters	Chou *et al.* (1978)	Yes	Tested 23 petrochemicals (including 5 aromatic compounds) for their susceptibility to anaerobic degradation.
	Yang *et al.* (1980)	No	Tested the effects of cyanide and chloroform on acetate-fermenting cultures.
	Suidan *et al.* (1981)	Yes	Activated carbon used as a support in reactors receiving synthetic wastewaters containing catechol and o-cresol.

Table 2.3 (cont.)

Reactor Type	Reference	Phenolics specifically used as substrate	Comments
	Khan et al. (1981)	Yes	Synthetic wastewater containing phenol fed to three anaerobic columns in series. Activated carbon served as the support.
	Rees and King (1981)	Yes	Columns of Lower Greensand (80% sand and 20% clay) were perfused with phenol solutions to mimic the situation of leachates from landfill site passing through the underlying porous rock.
	Dague (1981)	Yes	Wastewaters, high in phenols, from pyrolysis processes were mixed with domestic primary sludge and tested for toxicity and treatability.
Fluidized bed	Switzenbaum and Jewell (1980)	No	Aluminum oxide particles (500 um in diameter) used as support in 1-L reactor treating synthetic waste with glucose as main organic component.
	Jewell et al. (1981)	No	Polyvinyl chloride particles and expanded ion-exchange resin (<1 mm diameter) used as support. Settled domestic sewage fed into 1-L reactor.
	Hakulinen and Salkinoja-Salonen (1981)	Yes	Anaerobic fluidized bed in series with a trickling filter used to treat effluent from the bleaching of kraft pulp. ^{14}C-pentachlorophenol added to influent was not degraded anaerobically but gave ^{14}C-carbon dioxide from the aerobic reactor.
Phase-Separation	Borchardt (1971)	No	Two chambers of the reactor were separated by a dialysis membrane. The non-methanogenic chamber was fed raw domestic sludge.
	Ghosh and Klass (1977)	No	Studied phase separation by kinetic control. Kinetic data gathered for a variety of substrates including glucose, cellulose, sewage sludge and selected volatile acids.
	Massey and Pohland (1978)	No	Kinetic control used for phase separation. Both chambers were operated with and without biomass recycle. Substrates used in separate experiments were glucose and wastewater from a confectionary manufacturer.

sludge return, and attached growth systems such as anaerobic filters and fluidized beds) can provide very stable microbial populations. These processes minimize the hydraulic retention time and therefore reduce required reactor size. Jewell et al. (1981) have indicated that the fluidized bed is superior to the anaerobic filter because clogging is not a problem and nutrient diffusion is less limiting.

The phase separation method provides two chambers for microbial growth. The environmental conditions in the first chamber are optimum for the non-methanogens while those in the second chamber are optimum for the methanogens. Although this mode of operation appears to offer some advantages there are relatively few reports on the application of this method to anaerobic degradation.

2.3 Anaerobic Microbial Metabolism of Aromatic Compounds

Although this research project has addressed the methanogenic fermentation of phenolic compounds, the literature review deals with the anaerobic metabolism of aromatic compounds in general. This broader scope was chosen for two reasons.

First, phenolics and non-hydroxylated aromatics have been shown to be degraded in the absence of oxygen without the production of methane (Neufeld et al., 1980). A knowledge of such microbial activities would be beneficial in designing a two stage process (Borchardt, 1971; Pohland

and Ghosh, 1971; Massey and Pohland, 1978) which might overcome suspected phenolic toxicity to methane bacteria. Such metabolic activities might cleave the aromatic ring of phenolics which cannot be degraded in a single phase methanogenic fermentation. The resulting organic acids would be more amenable to methanogenic fermentation in another reactor.

Second, there are similarities in the degradation pathways of phenolic and non-phenolic aromatics. For example, methoxybenzoic acids have been shown to be converted to their corresponding hydroxybenzoic acids (which are phenolics) and then to benzoic acid prior to ring saturation and cleavage (Balba et al., 1979).

The topic of anaerobic degradation of aromatic compounds was reviewed several years ago by Evans (1977). This is updated in the following section.

2.3.1 Studies Using Pure Substrates

Most studies of the anaerobic microbial metabolism of aromatics have been done using a pure substrate fed to mixed cultures. These studies determine whether the substrate was susceptible to methanogenic fermentation. In some cases, pure substrates have been fed to pure cultures incubated anaerobically in the presence of nitrate which serves as a terminal electron acceptor. Often the facultative anaerobe used in these studies can grow on the substrate under both aerobic and anaerobic conditions. For example, Taylor et al.

(1970) have shown that different intermediates were produced by the same organism when grown aerobically or anaerobically. Therefore, different biochemical pathways must be operative in the presence and absence of oxygen. Other pure culture, pure substrate systems involve purple non-sulfur bacteria, the *Rhodospirillaceae*. Some of these are able to grow anaerobically in the light by photosynthetic means using simple aromatics as their sole carbon sources (Dutton and Evans, 1969).

The identification of intermediates of aromatic degradation has lead to proposed biochemical pathways for these substrates. All three modes of degradation (i.e. methanogenic fermentation, nitrate respiration, and photometabolism) involve the initial reduction of the ring prior to its cleavage. Benzoic acid, for example, is reduced to cyclohexane carboxylic acid and then the ring is broken as shown in Figure 2.1. This is in contrast with the aerobic method of degradation in which the ring is cleaved prior to reduction, producing a diene. (Dagley, 1967; Taylor et al., 1970). These two pathways (Figure 2.1) are representative but are not the only mechanisms of benzoic acid degradation.

Table 2.4 summarizes much of the literature dealing with metabolism of pure substrates by anaerobic microbial cultures. The 49 compounds considered here have been grouped under the following 10 headings: unsubstituted phenol, alkyl phenols, polyhydroxyphenols, methoxyphenols, chlorophenols, nitrophenols, aromatic amino acids, benzoic acid and its

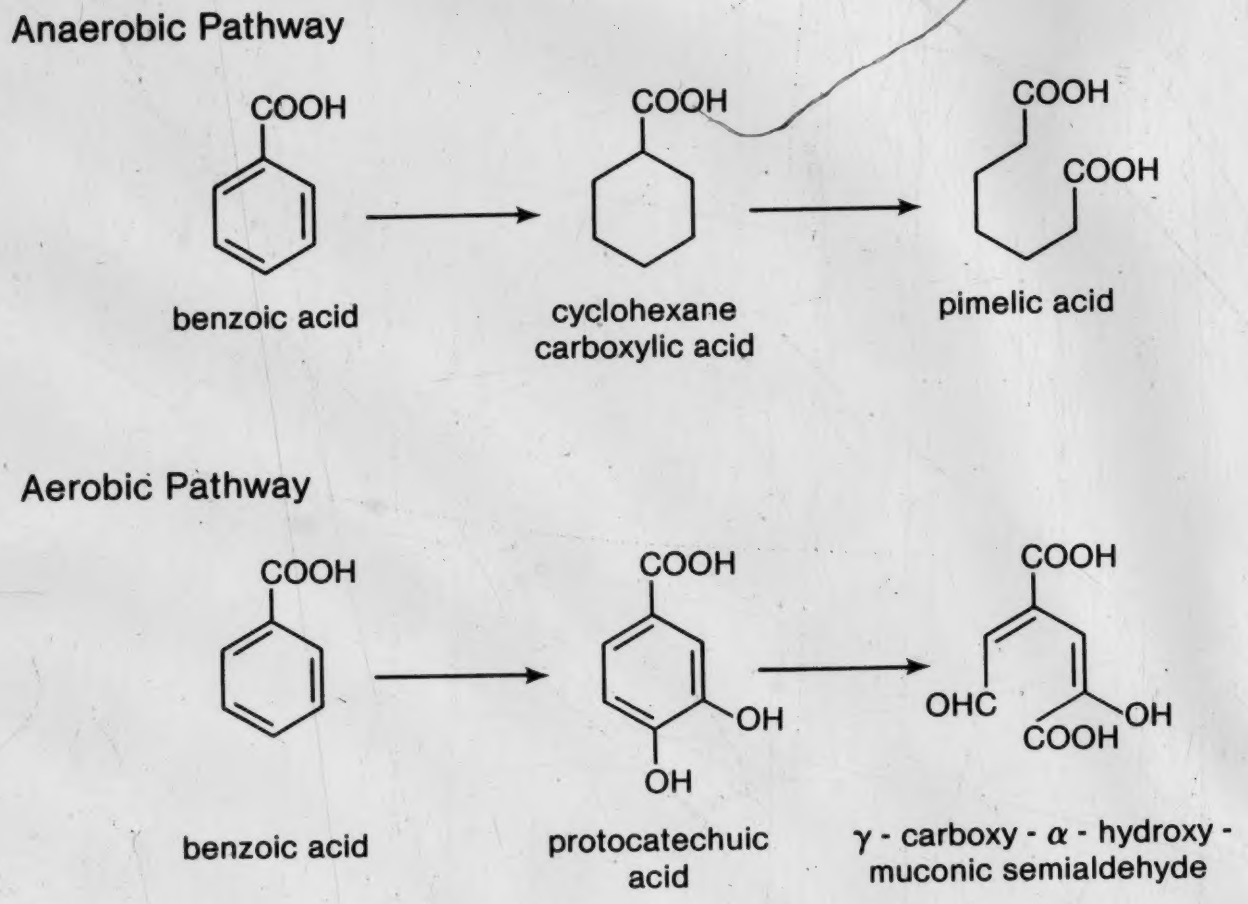

Anaerobic Pathway
COOH
benzoic acid
COOH
cyclohexane carboxylic acid
COOH
COOH
pimelic acid
Aerobic Pathway
COOH
benzoic acid
COOH
OH
OH
protocatechuic acid
COOH
OHC
COOH
OH
γ - carboxy - α - hydroxy - muconic semialdehyde

Figure 2.1 Comparison of some intermediates formed during anaerobic and aerobic microbial degradation of benzoic acid.

Table 2.4 Anaerobic metabolism of aromatic compounds

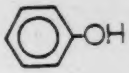
GROUP	COMPOUND	REFERENCE	COMMENTS
Unsubstituted Phenol			
	Phenol 	Tarvin and Buswell (1934)	Phenol found as transient intermediate in methane fermentation of tyrosine.
		Chmielowski *et al*. (1965)	Methane produced after 20 day lag. Fermentation continued when concentration increased to 1 500 mg/L.
		Chmielowski and Wasilewski (1966)	Unidentified alicyclic compounds formed as intermediate in methane fermentation. Catechol, the intermediate in aerobic degradation of phenol, was not formed.
		Bakker (1977)	Mixed culture in presence of nitrate produced cyclohexanol, cyclohexanone, and *n*-hexanoic acid. Methane not produced.
		Healy and Young (1978)	Hungate serum bottle method. Methane from approx. 300 mg/L phenol after 2.5 weeks.
		Healy and Young (1979)	Methanogenic cultures enriched on p-hydroxybenzoic acid could also ferment phenol.
		Neufeld *et al*. (1980)	Determined kinetic constants for the non-methanogenic fermentation of phenol in a mixed reactor.
		Khan *et al*. (1981)	Anaerobic activated carbon filter operated for 735 days with phenol as sole carbon source. Methane production observed.

Table 2.4 (cont.)

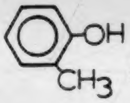
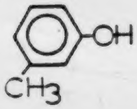
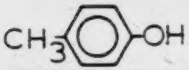
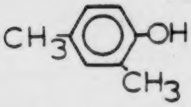
GROUP	COMPOUND	REFERENCE	COMMENTS
Alkyl phenols			
	o-cresol 	Chmielwoski et al. (1965)	Not fermented to methane
		Suidan et al.(1981)	Anaerobic activated carbon filter fed o-cresol did not produce gas. Another fed both glucose and o-cresol - methane production from the sugar was not inhibited by 256 mg/L o-cresol.
		Boyd et al. (1983)	Persistent over an incubation period of 8 weeks in batch cultures.
	m-cresol 	Chmielowski et al. (1965)	Not fermented to methane.
		Boyd et al. (1983)	Completely degraded within 7 weeks in batch cultures with methane production in excess of 90% of theoretical.
	p-cresol 	Chmielowski et al. (1965)	Methane produced after 12 day lag. Fermentation continued when p-cresol concentration increased to 1 700 mg/L.
		Chmielowski and Wasilewski (1966)	In a reactor adapted to the fermention of both p-cresol and resorcinol, p-cresol was degraded more slowly than resorcinol.
		Balba et al. (1981)	Methane produced with phenol and cyclohexanol as intermediates.
	2,4-dimethylphenol 	Chmielowski et al. (1965)	Not fermented to methane.

Table 2.4 (cont.)

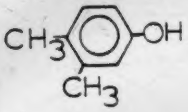
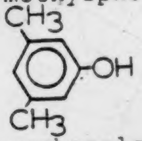
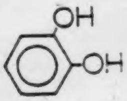
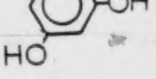
GROUP	COMPOUND	REFERENCE	COMMENTS
	3,4-dimethylphenol 	Chmielowski *et al*. (1965)	Not fermented to methane.
	3,5-dimethylphenol 	Chmielowski *et al*. (1965)	Not fermented to methane.
Polyhydroxyphenols			
	catechol 	Chmielowski *et al*. (1965)	Not fermented to methane.
		Healy and Young (1978)	Methane from approx. 300 mg/L substrate after 4.5 weeks.
		Healy and Young (1979)	Methane from approx. 300 mg/L substrate after 21 days.
		Balba and Evans (1980a)	Degradation intermediates include *cis*-benzenediol, (*cis*-1,2-dihydroxy-3,5-cyclohexadiene) phenol, cyclohexanol and cyclohexanone.
		Suidan *et al*. (1980)	Anaerobic activated carbon filter operated for 490 days with catechol as sole carbon source. Methane production observed.
		Suidan *et al*. (1981)	Anaerobic activated carbon filter operated for 617 days with catechol as sole carbon source. Methane production observed.
	resorcinol 	Chmielowski *et al*. (1965)	Methane produced after 15 day lag. Fermentation continued when resorcinol concentration increased to 1 600 mg/L.

Table 2.4 (cont.)

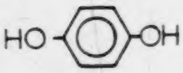
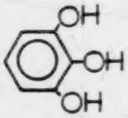
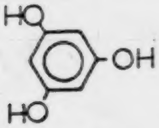
GROUP	COMPOUND	REFERENCE	COMMENTS
		Chmielowski and Wasilewski (1966)	In reactor adapted to fermentaton of both p-cresol and resorcinol, resorcinol was degraded more quickly than p-cresol.
		Chou *et al.* (1978)	Metabolized with production of gas from batch culture containing approx. 500 mg/L.
	hydroquinone 	Chmielowski *et al.* (1965)	Not fermented to methane.
		Chou *et al.* (1978)	Metabolized with production of gas from batch culture containing approx. 500 mg/L.
	pyrogallol 	Chmielowski *et al.* (1965)	Methane produced after 9 day lag. Fermentation continued when pyrogallol concentration increased to 1 700 mg/L.
	phloroglucinol 	Chmielowski *et al.* (1965)	Methane produced.
		Williams and Evans (1975)	Used anaerobically by *Moraxella* sp. by nitrate respiration.
		Whittle *et al.* (1976)	*Rhodopseudomonas gelatinosa* photometabolized substrate with production of three unidentified acids.
		Tsai *et al.* (1976)	Pure culture studies with a non-methanogen. One mole substrate give 2 moles acetate and 2 moles CO_2.
		Patel *et al.* (1981)	Studies with partially purified enzyme from a non-methanogen. Resorcinol is an early intermediate of degradation.

Table 2.4 (cont.)

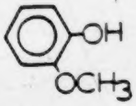
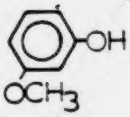
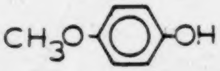
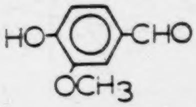
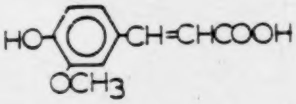
GROUP	COMPOUND	REFERENCE	COMMENTS
Methoxyphenols			
	o-methoxy-phenol 	Boyd *et al.* (1983)	Completely degraded within 2 weeks in batch cultures. 1,2-Dihydroxybenzene (catechol) was the initial intermediate. Methane was produced.
	m-methoxy-phenol 	Boyd *et al.* (1983)	Completely degraded within 1 week in batch cultures. 1,3-Dihydroxybenzene (resorcinol) was the initial intermediate. Methane was produced.
	p-methoxy-phenol 	Boyd *et al.* (1983)	Completely degraded within 1 week in batch cultures. 1,4-Dihydroxybenzene (hydroquinone) was assumed to be the initial intermediate but it was not detected. Methane was produced.
	vanillin 	Healy and Young (1979)	Methane produced after 12 day lag in medium with 300 mg/L. This culture was simultaneously adapted to ferment vanillic acid and syringaldehyde.
	ferulic acid 	Healy and Young (1979)	Methane produced after 10 day lag in medium with 300 mg/L substrate. This culture was simultaneously adapted to ferment cinnamic acid and vanillin.
		Healy *et al.* (1980)	Three morphological types of bacteria comprised more than 90% of the organisms in the methane producing enrichment culture which was simultaneously adpated to cinnamic acid, vanillic acid, and vanillin but not to phenol.

Table 2.4 (cont.)

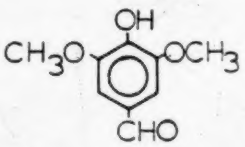
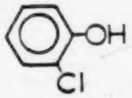
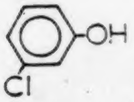
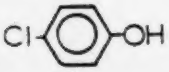
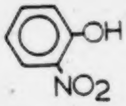
GROUP	COMPOUND	REFERENCE	COMMENTS
	syringaldehyde 	Healy and Young (1979)	Methane produced after 5 day lag in medium with 300 mg/L. This culture was simultaneously adapted to ferment syringic acid.
Chlorophenols			
	o-chlorophenol 	Boyd *et al.* (1983)	Completely degraded within 3 weeks in batch cultures. Dechlorinated to give phenol as initial intermediate. Methane was produced.
	m-chlorophenol 	Boyd *et al.* (1983)	Completely degraded within 7 weeks in batch cultures. Methane was produced.
	p-chlorophenol 	Boyd *et al.* (1983)	Persistent over a period of 8 weeks but completely degraded within 16 weeks. Methane was not detected
Nitrophenols			
	o-nitrophenol 	Boyd *et al.* (1983)	Completely degraded within 1 week in batch cultures. Methane was produced.

Table 2.4 (cont.)

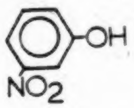
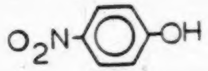
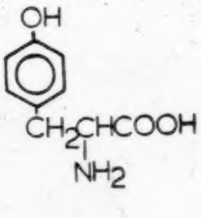
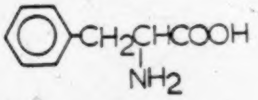
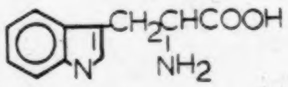
GROUP	COMPOUND	REFERENCE	COMMENTS
	m-nitrophenol 	Boyd *et al.* (1983)	Disappeared from batch culture medium within 1 week. Inhibited methane production during initial incubation period.
	p-nitrophenol 	Boyd *et al.* (1983)	Disappeared from batch culture medium within 1 week. Strongly inhibited methane production during initial weeks. Work with [U-ring^{14}C] substrate showed complete mineralization to $^{14}CO_2$ and $^{14}CH_4$.
Aromatic amino acids			
	tyrosine 	Tarvin and Buswell (1934)	Methane produced and phenol detected in medium.
		Balba and Evans (1980b)	Methane produced and the following intermediates found: p-hydroxyphenylacetate, phenylacetate, cyclohexylacetate, p-hydroxyphenylpyruvate, p-cresol and phenol.
	phenylalanine 	Balba and Evans (1980b)	Methane produced with phenylacetate as main intermediate.
	tryptophan 	Balba and Evans (1980b)	Methane produced and the following intermediates found: indol-3-ylacetate, indole, anthranilate, salicylate, benzoate and cyclohexanecarboxylate.

Table 2.4 (cont.)

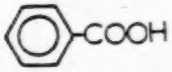
GROUP	COMPOUND	REFERENCE	COMMENTS
benzoic acid and its derivatives			
	benzoic acid	Tarvin and Buswell (1934)	Methane produced.
		Fina and Fiskin (1960)	Radioactive substrate showed carboxyl carbon goes mainly to carbon dioxide and carbon 1 goes to methane.
		Guyer and Hegeman (1969)	Proposed reductive pathway for photometabolism by *Rhodopseudomonas palustris*. Intermediates included cyclohexene-1-carboxylic acid, 2-hydroxycyclohexane carboxylic acid, 2-ketocyclohexane carboxylic acid and heptanedioic acid.
		Taylor et al. (1970)	Degraded by *Pseudomonas* PN-1 anaerobically in the presence of nitrate and also aerobically. The intermediates formed under aerobic growth conditions were not found during anaerobic growth.
		Williams and Evans (1975)	*Moraxella* sp. grew anaerobically on benzoate by nitrate respiration. Intermediates included 2-hydroxycyclohexanecarboxylic acid which lost the carboxyl carbon giving hexanedioic acid.
		Ferry and Wolfe (1976)	Fermentation of [U-ring^{14}C] benzoate gave 50% of the radioactivity as carbon dioxide and 50% as methane. Also showed that methane bacteria alone could not ferment benzoate.

Table 2.4 (cont.)

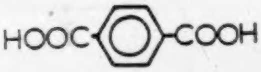
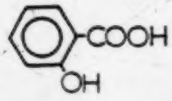
GROUP	COMPOUND	REFERENCE	COMMENTS
		Keith et al. (1978)	Methane produced. Intermediates prior to ring cleavage were cyclohexene-1-carboxylic acid and cyclohexane carboxylic acid. Fatty acids found were heptanoic, pentanoic, butyric, propionic and acetic.
		Shlomi et al. (1978)	Methane produced with the following acid intermediates detected: 2-hydroxycyclohexane carboxylate, 2-oxocyclohexane carboxylate, heptanedioic acid, hexanoate, butyrate, acetate.
		Healy and Young (1979)	Cultures able to produced methane from p-hydroxybenzoic acid or vanillic acid were simultaneously adapted to ferment benzoic acid.
	phthalic acid	Williams and Evans (1975)	Not utilized by *Moraxella* sp. incubated anaerobically with nitrate.
		Chou et al. (1978)	Metabolized with the production of gas.
	o-hydroxybenzoic acid	Chmielowski et al. (1965)	Methane produced after lag of approx. 14 days.
		Dutton and Evans (1969)	Not photometabolized by *Rhodopseudomonas* sp.
		Williams and Evans (1975)	Not utilized by *Moraxella* sp. incubated anaerobically with nitrate.

Table 2.4 (cont.)

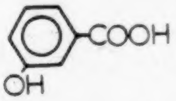
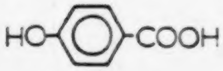
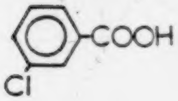
GROUP	COMPOUND	REFERENCE	COMMENTS
	m-hydroxy-benzoic acid	Chmielowski *et al.* (1965)	Methane produced.
		Dutton and Evans (1969)	Photometabolized by *Rhodopseudomonas* sp.
		Taylor *et al.* (1970)	Degraded by *Pseudomonas* PN-1 anaerobically in presence of nitrate.
		Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically nitrate.
	p-hydroxy-benzoic acid	Chmielowski *et al.* (1965)	Methane produced.
		Dutton and Evans (1969)	Photometabolized by *Rhodopseudomonas* sp.
		Taylor *et al.* (1970)	Degraded by *Pseudomonas* PN-1 anaerobically in presence of nitrate.
		Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically with nitrate.
		Healy and Young (1979)	Methane produced after 12 day lag in medium with 300 mg/L substrate. This culture was simultaneously adapted to ferment phenol and benzoic acid.
	3-chloro-benzoic acid 	Horowitz *et al.* (1983)	Dechlorinated to benzoic acid after lag period of 30 to 52 weeks. Methane production observed.

Table 2.4 (cont.)

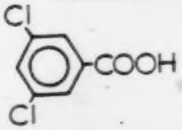
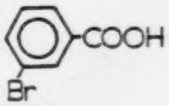
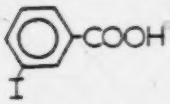
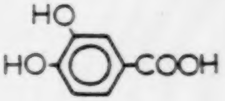
GROUP	COMPOUND	REFERENCE	COMMENTS
	3,5-dichlorobenzoic acid 	Horowitz et al. (1983)	Dechlorinated to 3-chlorobenzoic acid after lag period of 2 to 3 weeks. Methane production observed.
	3-bromobenzoic acid 	Horowitz et al. (1983)	Debrominated to benzoic acid after lag period of 0.2 to 1 week. Methane production observed.
	3-iodobenzoic acid 	Horowitz et al. (1983)	Deiodinated to benzoic acid after lag period of 1 to 3 weeks. Methane production observed.
	protocatechuic acid 	Taylor et al. (1970)	Degraded by *Pseudomonas* PN-1 incubated anaerobically in presence of nitrate but not degraded under aerobic conditions.
		Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically with nitrate.
		Healy and Young (1979)	Methane produced after 13 day lag in medium with 300 mg/L substrate. Cultures adapted to vanillic acid would also produce methane from protocatechuic acid.
		Balba et al. (1979)	Methane produced by benzoate fermenting culture. Two degradation pathways detected. The major route had catechol as an intermediate. The minor route had m-hydroxybenzoic acid and benzoic acid.

Table 2.4 (cont.)

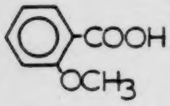
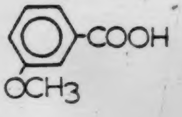
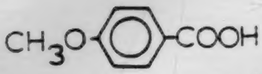
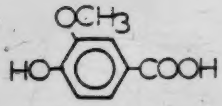
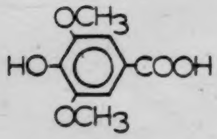
GROUP	COMPOUND	REFERENCE	COMMENTS
	o-methoxy-benzoic acid 	Balba *et al.* (1979)	Methane produced by benzoate fermenting culture after 1-month lag. o-Hydroxybenzoic acid accumulated which was degraded to benzoic acid.
	m-methoxy-benzoic acid 	Balba *et al.* (1979)	Methane produced by benzoate fermenting culture after 3-week lag. m-Hydroxybenzoic acid accumulated which was degraded to benzoic acid.
	p-methoxy-benzoic acid 	Balba *et al.* (1979)	Methane produced by benzoate fermenting culture after 3-week lag. p-Hydroxybenzoic acid accumulated which was degraded to benzoic acid.
	vanillic acid 	Healy and Young (1979)	Methane produced after 9 day lag in medium with 300 mg/L substrate. Cultures adapted to vanillic acid would also produce methane from syringaldehyde, syringic acid, vanillin, benzoic acid, catechol and protocatechuic acid.
	syringic acid 	Healy and Young (1979)	Methane produced after 2 day lag in medium with 300 mg/L substrate. Cultures adapted to syringic acid would also produce methane from syringaldehyde and vanillin.

Table 2.4 (cont.)

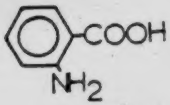
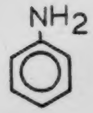
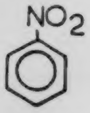
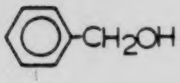
GROUP	COMPOUND	REFERENCE	COMMENTS
	anthranilic acid 	Balba and Evans (1980b)	Intermediate formed during methanogenic fermentation of tryptophan. Degraded to o-hydroxybenzoic, benzoic, cyclohexane carboxylic, cyclohex-1-ene carboxylic, adipic and other organic acids.
		Balba *et al.* (1981)	Aniline, phenol and cyclohexanol were major intermediates found in methane-producing culture.
miscellaneous monoaromatics			
	aniline 	Tarvin and Buswell (1934)	Was not fermented to methane.
		Chou *et al.* (1978)	Metabolized with the production of gas. Benzoate fermenting culture was simultaneously adapted to ferment aniline.
		Balba *et al.* (1981)	Found as an intermediate during the degradation of anthranilic acid in the presence of sulfate reducing bacteria.
	nitrobenzene 	Chou *et al.* (1978)	Metabolized with the production of gas.
	benzyl alcohol 	Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically with nitrate.
		Balba *et al.* (1981)	Methane produced.

Table 2.4 (cont.)

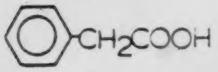
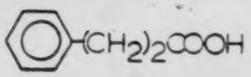
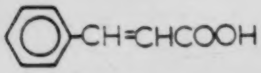
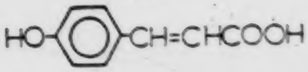
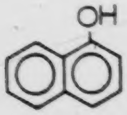
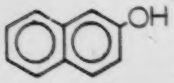
GROUP	COMPOUND	REFERENCE	COMMENTS
	phenylacetic acid	Tarvin and Buswell (1934)	Methane produced.
		Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically with nitrate.
		Balba and Evans (1979)	Methane produced by benzoate fermenting culture after a lag period of 18 days. Intermediates included cyclohexyldiene acetate, cyclohexylacetate and cyclohexanone. substrate. This culture was simultaneously adapted to ferment vanillic acid and syringaldehyde.
	β-phenylpropionic acid	Tarvin and Buswell (1934)	Methane produced.
		Taylor et al. (1970)	Degraded by *Pseudomonas* PN-1 in the presence of nitrate after a lag of approx. 10 days.
		Balba and Evans (1979)	Methane produced by benzoate fermenting culture with no lag period. Intermediates included benzoate, cyclohexanecarboxylate and hexanedioate.
	cinnamic acid	Tarvin and Buswell (1934)	Methane produced .
		Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically with nitrate.
		Healy and Young (1979)	Methane produced after 13 day lag in medium with 300 mg/L substrate. Culture adapted to ferulic acid would also produce methane from cinnamic acid.

Table 2.4 (cont.)

GROUP	COMPOUND	REFERENCE	COMMENTS
		Balba and Evans (1979)	Immediately utilized, with methane production, by cultures adpated to benzoate and β-phenylpropionate.
	p-hydroxy-cinnamic acid 	Williams and Evans (1975)	Degraded by *Moraxella* sp. incubated anaerobically with nitrate.
naphthols			
	1-naphthol 	Chmielowski *et al*. (1965)	Not fermented to methane.
	2-naphthol 	Chmielowski *et al*. (1965)	Not fermented to methane.

derivatives, miscellaneous monoaromatics, and naphthols. In this scheme, aromatics containing both hydroxy and carboxy groups have been classified as derivatives of benzoic acid rather than as phenols.

Horowitz et al. (1982) screened 78 aromatic compounds for their susceptibilities to degradation by anaerobic consortia from a fresh water lake sediment and from municipal digested sludges. Among these were 34 benzoic acid derivatives including various isomers of halogenated, methoxy, methyl, nitro and amino compounds; 21 phenolics with similar substitutents; seven phthalates; and 12 anilines. Forty-two of these compounds were metabolized. More detailed work with fewer of these compounds has been reported by Horowitz et al. (1983) and Boyd et al. (1983) and only this latter group of better studied compounds have been included in Table 2.4.

Other workers have challenged laboratory anaerobic cultures with a variety of phenolics to determine whether these were inhibitory to the methanogenic fermentation. Pearson et al. (1980) used various concentrations of phenol while Johnson (1981) tested 2-chlorophenol, 2-nitrophenol, 4-nitrophenol, 2,4-dichlorophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, and 2,4,6-trichlorophenol in their inhibition studies. Since these studies were testing for inhibition, rather than fermentability, those results have not been included in Table 2.4.

2.3.2 Anaerobic Treatment of Phenolic Wastewaters

Although Chmielowski and Kusznik (1966) reported the methanogenic fermentation of phenolic wastewaters 18 years ago, there have been very few reports on the subject since then. Research in this area has not been pursued because of the limited number of phenolic compounds which were perceived to be biodegradable under anaerobic conditions and because many of the wastewaters which contain phenolics also contain other toxic materials (e.g. CN^- and SCN^-) which complicate such studies. This section reviews the small amount of published information dealing with laboratory-scale treatment of phenolic wastewaters.

Chmielowski and Kusznik (1966) investigated the feasibility of treating three different types of phenolic wastewaters. They used a draw and feed procedure with 1 L of anaerobic inoculum in a sealed 3 L container. One wastewater was from a phenol-synthesizing plant, it contained mainly phenol and it was used both in its "raw" state with 17 000 mg/L phenol and after solvent extraction which left 920 mg/L phenol. The second wastewater came from the conversion of coal to heating gas and contained 5 645 to 7 750 mg/L phenolics. The third wastewater was from a coking process and it contained phenolics in the range of 5 320 to 7 270 mg/L.

Cultures which had been acclimated to pure phenol were each fed one of the wastewater types. Aliquots of the wastewaters added to the reactors gave phenolic concentrations between 220 and 1 000 mg/L in the cultures. The "raw" wastes from phenol-synthesis were readily fermented but those which had been solvent extracted contained too little substrate and the fermentation was hampered because of cell washout. Methane was produced from the coal-derived wastes in spite of the presence of non-fermentable phenolics in addition to fermentable compounds such as phenol and p-cresol. Prior acclimation of the sludge to phenol degradation was essential since attempts to directly acclimate anaerobic sludge to treat the coal-derived wastes were unsuccessful.

Wastewater from an oil shale retorting process was treated in a laboratory-scale anaerobic digester by Ossio and Fox (1980). Digested sludge from a treatment plant receiving both municipal and industrial wastes was acclimated by stepwise increases in the proportion of retort water fed to the laboratory digester. Ammonia removal and neutralization of the wastewater were found to be essential. Carbon dioxide was used to reduce the pH after ammonia stripping at pH 11. Prior use of H_2SO_4 during the early stages of the study was thought to produce sulfide toxicity through the activity of sulfate reducing bacteria and was therefore discontinued. The addition of the nutrients calcium, magnesium and phosphorus was required to achieve a 65 to 70% COD removal and 90% BOD_5 removal.

Liquid wastes from the pyrolysis of municipal refuse were subjected to anaerobic degradation in a variety of experiments reported by Dague (1981). The major organic components of the wastewater were phenols, alcohols and amines. It also contained polynuclear aromatics, and heavy metals including Zn, Pb, Cr, and Cd. Both suspended growth and attached growth reactors were used. In all cases, the pyrolysis wastewater had to be diluted with a more readily degradable waste such as domestic primary effluent. Also, acclimation to these wastes was achieved only by gradually increasing the proportion of pyrolysis wastewater in the influent mixture. In the anaerobic contact mode (solids retention time of 15 days and a hydraulic retention time of five days), the suspended growth method would handle a feed which contained 30% of its COD from the pyrolysis wastewater and would remove up to 70% of the COD from that source. The two 1.22 m x 0.14 m diameter anaerobic filters were operated in series and handled the same strength influent with the same COD removal efficiency as the suspended growth reactor.

In their comparison of two different packing media (anthracite coal and activated carbon) in "completely mixed" anaerobic filters, Khan et al. (1982) fed diluted and nutrient-supplemented aircraft paint-stripping wastes to reactors which had been acclimated to phenol fermentation. The influent wastewater contained 500 mg/L phenol and 1 460 mg/L dissolved COD. Stable conditions were attained after 22 days of operation with the new wastewater. Over the next 26 days of operation, the anthracite coal packed column reduced the phenol and dissolved COD concentrations by 54% and 81%, respectively, while the activated carbon packed column reduced these parameters by 82% and 90%, respectively. The rates of methane production were 0.36 and 0.67 L/day in the anthracite and activated carbon columns, respectively.

Cross et al. (1982) used an anaerobic reactor system consisting of two 183 cm x 10 cm (internal diameter) columns in series. The first was packed with Raschig rings and the second contained activated carbon. Settled digested sludge was used as an inoculum for the first column. Wastewater from a coal gasification plant containing 5 600 mg/L phenol with a COD of 11 900 mg/L was diluted to 10% (V/V) with phosphate solution and fed to the system. COD removals were near 80% while phenol and cresol removals were in excess of 90% and 99%, respectively. Only 10% - 20% of the phenol removal and approximately 58% of the cresol removal occurred in the first column. The majority of the methane produced (86%) came from the second column. The total quantity of methane produced was near that expected from the degradation of the influent phenol.

Suidan et al. (1983) operated laboratory scale anaerobic filters followed by an activated sludge nitrification unit to treat coke-oven wastewater and then coal gasification wastewater. During their initial studies a berl-saddle-packed anaerobic filter was fed 1 000 mg/L glucose and 5% coke-oven wastewater supplemented with nutrients. Vigorous methane production was observed during this 61-day period. However, when the influent was switched to a nutrient-supplemented 10% dilution of the coal gasification wastewater, and the glucose eliminated from the feed, methane production decreased markedly within 27 days. An expanded bed granular activated carbon anaerobic filter was then placed between the berl-saddle-packed unit and the nitrification unit. This system was operated for 118 days on nutrient-supplemented 10% coal gasification wastewater. During this time, the berl-saddle-packed unit removed little or no total organic carbon (TOC) or dissolved organic carbon (DOC) while the activated carbon filter removed most of the TOC and DOC. Over the first 63 days when both anaerobic systems were in use, much of the organic removal was by adsorption to the activated carbon. However between days 63 and 91, vigorous methane production was observed along with a marked improvement in the effluent quality. After 118 days operation, the berl-saddle-packed unit was removed leaving the activated carbon unit as the sole anaerobic reactor. This was maintained for a further 184 days. Methane production remained stable while the concentrations of organic material in the effluent slowly increased. This material was thought to be non-biodegradable compounds which were escaping because of the gradual loss of the adsorptive capacity of the activated carbon.

Suidan et al. (1983) also provided analyses of specific phenolics in the influent and effluent of their anaerobic activated carbon unit. Analysis for one specific day indicated that the removal of phenol (originally at

207 mg/L) was >99.9%. Removal of o-cresol, m/p-cresol (these two isomers were not resolved by their analytical method) and the dimethylphenols were 75%, 87% and 98%, respectively. These removals were attributed to a combination of biodegradation and adsorption.

2.4 Analytical Methods

In order to study the microbial degradation of any compound, one must be able to measure the loss of that substrate, and/or detect known intermediates or metabolic end products. In the present study, phenolics were the test substrates, and methane was the major end product. Analytical methods for measuring these two parameters are reviewed in the following two sections.

2.4.1 Analysis of Phenolics

As a general class, phenolics are often estimated by the 4-aminoantipyrine method given by APHA (1980). Several GC methods using direct aqueous injections are also given by APHA (1980) which allow the identification (based on retention time) and quantitation of specific phenolics. Since the major phenolics of interest in this project were the alkyl substituted compounds, the literature cited deals mainly with this group.

The direct aqueous injection technique is extremely convenient to use because there is no time spent on sample preparation. Barker and Malo (1967) reviewed a wide range of liquid phases and solid supports used for the GC quantitation of a variety of phenolics including phenol and the three isomers of cresol. The recoveries of these four compounds from spiked sludge were shown to be between 96% and 99% (Baird et al., 1974) although the m- and p- isomers were not resolved by their column (4% dinonylphthalate on Chromosorb G). Ho et al. (1976) used a column packed with Tenax GC to detect five isomers of dimethylphenol, the cresols, ethylphenol, and phenol in by-product water from fossil fuel conversion processes. Again the m- and p- isomers of cresol were not resolved. Tenax GC coated with 5% polymetaphenyl ether (Bartle et al., 1977) gave more symmetrical peaks (i.e. less tailing) than did the uncoated polymer and improved the sensitivity of the method.

Since the quantitative solvent extraction of phenolics from aqueous samples is difficult and time consuming, Coutts et al. (1979) have developed a method of direct acetylation in aqueous solution. The resulting acetate derivatives are easily extracted into an organic solvent for GC analysis.

Several workers have demonstrated excellent resolution of many alkyl phenolics, including m- and p- cresol, in organic solvents using capillary GC columns of glass (Guenther et al., 1981) and fused silica (Dandeneau et al., 1979).

High performance liquid chromatography (HPLC) has also been used to analyze alkyl phenolics. Schabron et al. (1978) report retention data for 35 phenolics on five different chromatography columns. Reverse-phase HPLC was used by Sparacino and Minick (1980) to quantitate phenol, cresols, and dimethylphenols (xylenols) in coal gasifier condensate. However, their method could not resolve the isomers of a given alkyl phenol. Ogan and Katz (1981) used both ultraviolet and fluorescence detectors in their study of 19 alkyl phenols. The latter detector was the more sensitive and selective when used for the analysis of a complex coal gasification sample.

Often, in studies of the degradation of a pure substrate in a microbial culture, phenolics are quantitated on the basis of their absorption of ultraviolet radiation at characteristic wavelengths (Healy and Young, 1978 and 1979; Khan et al., 1981; Suidan et al., 1981). The ultraviolet absorption characteristics for 30 phenolic compounds in 0.4 N NaOH have been reported by Eisenhauer (1964).

2.4.2 Methane Analysis

The analysis for methane using gas-solid chromatography can be done with a variety of porous polymers such as the Chromosorb or Porapak series or Molecular Sieves, alumina or silica gel. Thompson (1977) lists retention data from many of these packing materials under different column operating conditions. Methane can be detected by either of the most common GC detectors - the thermal conductivity detector or the flame ionization detector.

Gases found in the headspace of an anaerobic culture include methane, carbon dioxide, water, nitrogen, hydrogen sulfide, and hydrogen. Of these, the flame ionization detector responds to only methane (Sullivan, 1977). Thus, almost any GC column can be used to analyze for methane when this detector is used. The column need not separate the gases because the detector responds only to methane.

3. MATERIALS AND METHODS

This section describes the general techniques for medium preparation and the analytical methods which were used throughout this study. More specific experimental designs are given in each chapter where particular aspects of the investigation are considered.

3.1 Anaerobic Batch Culture Methods

All fermentability and inhibition studies were done using the serum bottle modification of the Hungate method (Miller and Wolin, 1974). The headspace gas in the culture bottles was 30% CO_2 in N_2 (Matheson) which had been scrubbed free of O_2 by passage through a heated glass column packed with copper ribbons (Hungate, 1969). When required, the oxidized copper was reduced by a flow of 10% H_2, 5% CO_2 in N_2 (Matheson) through the hot column. Prior to the addition of medium to serum bottles or Hungate type culture tubes (Bellco Glass, Inc.) these containers were flushed with O_2-free 30% CO_2 in N_2 flowing at approximately 2 L/min for 40-60 s.

Stock solutions of inorganic salts, vitamins, a redox indicator (resazurin), 2-methyl-n-butyric acid and the reducing agent (sulfide) used in the culture medium were prepared as outlined in Table 3.1. These were combined in the proportions given in Table 3.2 and were boiled for 2 min prior to the addition of 20 mL anaerobic sludge filtrate (passed through Whatman #1) as a nutrient supplement.

Table 3.1. Stock solutions used in growth medium.

Solution	Components	Concentration in distilled water (g/L)
Mineral I(1)	NaCl	50
	$CaCl_2.2H_2O$	10
	NH_4Cl	50
	$MgCl_2.6H_2O$	10
Mineral II	$(NH_4)_6Mo_7O_{24}.4H_2O$	10
	$ZnSO_4.7H_2O$	0.1
	H_3BO_3	0.3
	$FeCl_2.4H_2O$	1.5
	$CoCl_2.6H_2O$	10
	$MnCl_2.4H_2O$	0.03
	$NiCl_2.6H_2O$	0.03
	$AlK(SO_4)_2.12H_2O$	0.1
Vitamin B	Nicotinic acid	0.1
	Cyanocobalamine	0.1
	Thiamine	0.05
	p-aminobenzoic acid	0.05
	Pyridoxine	0.25
	Pantothenic acid	0.025
Phosphate	KH_2PO_4	50
Resazurin		0.1
2-methyl-n-butyric acid(2)		102
Sulfide(3)	$Na_2S.9H_2O$	25

(1) Dissolved in 0.01 M HCl rather than distilled water

(2) Adjusted to pH 6.5

(3) Prepared in small aliquots in freshly boiled water just prior to use.

Table 3.2 Composition of growth medium.[1]

Solution	Volume (mL)
Distilled water	70
Mineral I	1
Mineral II	0.1
Vitamin B	0.1
Phosphate	1
Resazurin	1
2-methyl-n-butyric acid	0.1

---

[1] 20 mL filtered anaerobic sludge added after the above solutions have been boiled.

Boiling continued for 1 min and 0.34 g $NaHCO_3$ was added. The medium was allowed to cool while O_2-free 30% CO_2 in N_2 was being bubbled through the liquid until its pH was 7 ± 0.1.

Typically batch cultures contained 4 mL medium, 1 mL phenolic solution and 5 mL anaerobic sludge as an inoculum. In some cases, these proportions were altered and a more concentrated medium was prepared by decreasing the volume of water combined with the other solutions outlined in Table 3.2. The new volume of water was calculated according to the following expression:

$$[(93.3)(n/4)] - (20 + 3.3) \text{ mL} \tag{3.1}$$

where n is the volume of medium (mL) to be dispensed for a 10 mL culture ($n \geq 1$); 3.3 is the total volume of the solutions in Table 3.2 excluding water; 20 mL accounts for the filtered sludge; and 93.3 is the total volume of all components in the regular strength medium.

Solutions of phenolic were prepared in distilled water at concentrations ten times their desired concentrations in the test cultures. A 1 mL aliquot of the phenolic solution was added to a serum bottle and O_2-free 30% CO_2 in N_2 was bubbled through the aliquot for about 3 min at approximately 100 mL/min. Then 4 mL medium were added to the bottle which was then sealed and autoclaved for 15 min at 121°C. Just prior to inoculation, 0.1 mL of the sulfide solution was injected into each bottle and upon reduction, the medium turned from pink to straw-yellow. If the substrate was thought to be heat labile, it was injected into the culture after inoculation.

For any given set of test conditions, batch cultures were set up in triplicate. Similarly, control cultures which contained aliquots of distilled water rather than the test substrate solution were set up in triplicate. Methane production (or substrate loss) in the test cultures was compared to that in the control cultures to determine what effect the test condition had on the microbial activity in the cultures.

3.2 Analytical Methods

3.2.1 Substrate Analyses

The GC method of Bartle _et_ _al_. (1977) was used for quantitative analyses of phenolic substrates in the anaerobic cultures and industrial wastewaters. Prior to analysis, the culture bottles were inverted for approximately 20 min to allow the solids to settle. Then a 3 μL aliquot of the medium was removed by means of a Hamilton 10 μL syringe. This was injected into a 4.6 m x 3.2 mm column of premium grade stainless steel (Supelco, Inc.) packed with Tenax GC 60/80 mesh (Alltech Assoc) which had been coated with 5% Polyphenyl ether 6 ring (Chromatographic Specialties). The column was in a Varian model 1700 GC equipped with a flame ionization detector operating with hydrogen and air flow rates of 30 and 300 mL/min, respectively. Nitrogen was used as a carrier gas at 17 mL/min. The oven, injection port and detector temperatures were 200°, 250° and 350°C, respectively. Peak areas were measured using a Hewlett-Packard model 3390A reporting integrator.

Although the Polyphenyl ether coating gave less tailing than the uncoated Tenax GC method of Ho et al. (1976), some residual tailing caused anomalies in peak area recording by the integrator. To minimize the errors caused by this inconsistency, two standard curves were prepared for each substrate, one for low concentrations (up to 100 mg/L) and one for high concentrations (100 - 800 mg/L). The substrate concentrations in the culture fluids were then determined using the external standard method based on peak areas.

3.2.2 Methane Analysis

Three different GC methods were used to determine the methane concentration in the headspace gas in the culture bottles. The method used on any occasion, depended upon the availability of the required instrumentation. A Varian-Aerograph model 700 equipped with a thermal conductivity detector was fitted with a 3.0 m x 9.5 mm Flexible Pentube I column packed with Porapak R (60/80 mesh). Helium was used as the carrier gas at a flow rate of 80 mL/min. The oven, injector and detector temperatures were 58°, 40°, and 38°C, respectively. Chromatograms were recorded on a Hewlett-Packard 7101B recorder and peak heights were used for quantitation.

The second GC system was a 2.1 m x 3.2 mm stainless steel column packed with 4% H_3PO_4 on Porapak Q (100/120 mesh) installed in a Varian model 1700 equipped with a flame ionization detector. Nitrogen was used as the carrier gas at a flow rate of 49 mL/min. The oven, injector and detector temperatures were 25°, 200° and 350°C, respectively. The hydrogen and air flow rates into the detector were 30 and 300 mL/min, respectively. Peaks were recorded and integrated using a Hewlett-Packard 3390A integrator.

The third system was a MicroTek model MT220 GC equipped with a flame ionization detector. A 1.8 m x 2 mm ID glass column packed with GP 10% SP-1000/1% H_3PO_4 on Chromosorb W AW (100/120) was used with a nitrogen carrier gas flow of 30 mL/min. The injection port and oven temperatures were 25°C while the detector temperature was 145°C. A Hewlett-Packard 3390A integrator was used for peak area determination.

Quantitative standard curves were prepared for each set of gas analyses. Known volumes of methane (Matheson, C.P./99.0% min.) were injected into sealed serum bottles. The actual concentration of methane in the rigid container was calculated using the following equation (Nelson, 1971):

$$\text{Percent } CH_4 = \frac{100 \text{ x vol of } CH_4 \text{ added}}{\text{vol of } CH_4 \text{ added + vol of serum bottle}} \qquad (3.2)$$

3.2.3 Gas Volume Measurements

In some experiments, the absolute volumes of gas and/or methane produced were determined by measuring the total volume of gas in the culture bottle and in the latter case, the proportion of methane in the gas phase. A novel apparatus was designed and used for these measurements.

3.2.3.1 Description of Gas Volume Measuring Apparatus

The apparatus is shown in Figure 3.1 and consists of a suitable size hypodermic needle (A) attached to 3-way stopcocks (B and C) (Propper Manufacturing Co., New York) which were modified (by simply cutting away a small plastic stop) to allow the plug to rotate 360°. As supplied by the manufacturer, the rotation is only 270° and only two of the three outlets of the stopcock could be open at one time. This modification allows any two or all three outlets to be open simultaneously. Glass syringes, lubricated with silicone vacuum grease, are attached to the upper 3-way stopcock and are supported by laboratory clamps. These syringes may be any convenient size and can be quickly replaced if required. Figure 3.1 shows a 30 mL (D) and a 10 mL syringe (E). The side arm of the lower stopcock is connected to a simple water manometer by a short piece of Tygon tubing (F). In the event that water from the manometer is accidently drawn back into the connecting tubing it can be readily seen through the clear tubing. Any water therein should be removed or the tubing replaced prior to the next use of the apparatus since its presence adversely affects

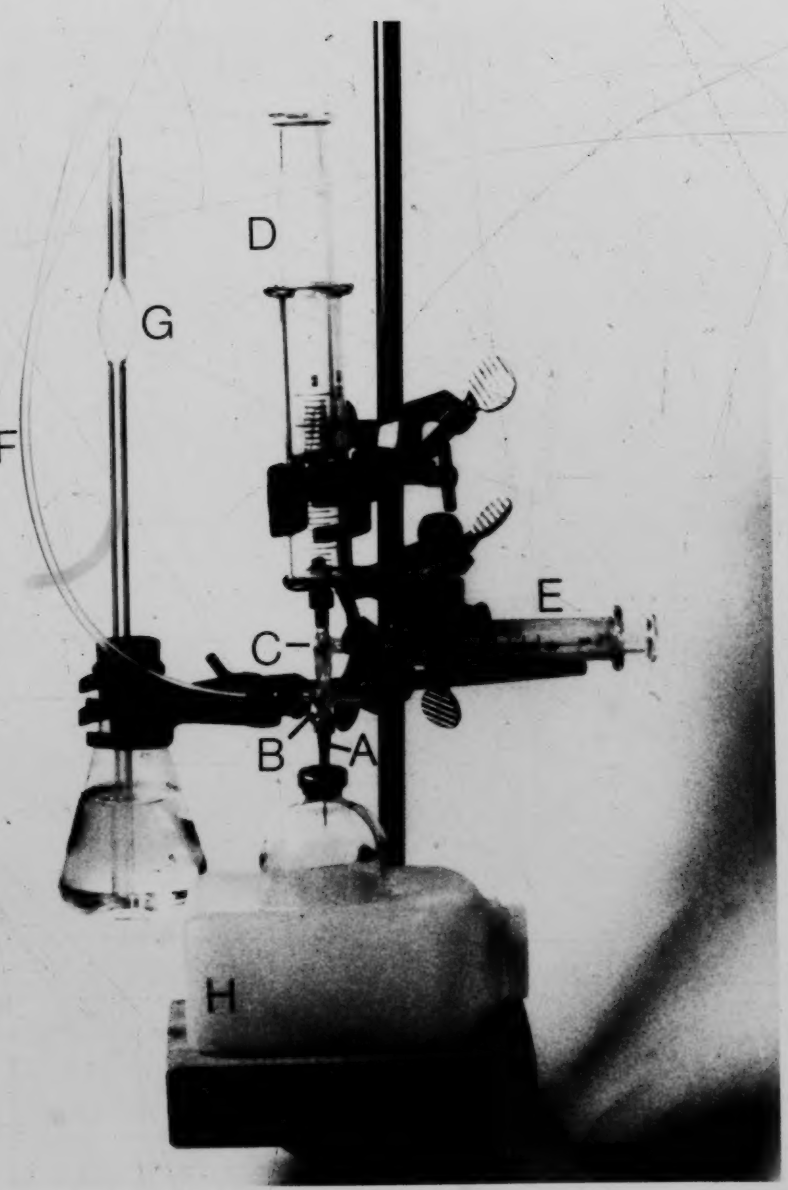


Figure 3.1 Apparatus for measuring the volume of gas produced by methanogenic cultures in serum bottles (see text for details).

the overall performance of the device. The manometer consists of a 10 mL volumetric pipette (G) inserted through a 2-hole stopper into an Erlenmeyer flask of water. The bulb in the pipette serves as a reservoir which minimizes the chance of water getting into the connecting tubing.

Also shown in Figure 3.1 is a plastic reagent bottle (H) in which a hole, slightly larger than the diameter of the culture bottle, has been cut. This serves as an insulator so that heat from the experimenter's hand does not affect the gas volume measurement.

After the apparatus was assembled and prior to its use, all of the connections were tested to ensure they were gas tight. This was done by drawing 8 mL air into syringe D and then inserting the needle deep into a rubber stopper. Stopcock B was positioned so that all three arms were open and a pinch clamp was placed on the Tygon tubing. The syringes, stopcocks and needle were immersed in water and the plunger depressed to give a gas volume of 2 mL and a pressure of near 4 atmospheres. No leaks were detected. The gas was then transferred to syringe E and the procedure repeated. This test is routinely done to ensure that no leaks have developed which would result in the loss of headspace gas or which could lead to the introduction of O_2 into the anaerobic culture.

3.2.3.2 Use of the Apparatus

Prior to making a gas measurement, the serum bottle culture is removed from the incubator and allowed to reach ambient temperature (1.5 h for a 50 mL culture in a 158 mL serum bottle taken from a 37°C incubator). The entire apparatus is flushed repeatedly with O_2-free 30% CO_2 in N_2 and stopcock B is positioned so the arm to syringe E is closed. The culture bottle is then quickly placed in the insulator (H) and the bottle is moved upward to insert the needle through the stopper. A small piece of wood (I) is placed on the ring clamp to support the weight of the culture bottle.

The plunger in syringe D is drawn out to accommodate the gas volume which is in excess of the headspace volume in the culture bottle. If the volume of syringe D is insufficient, stopcock C is opened so that syringe E can also be used. With practice the user can feel when the gas pressure within the syringes and the culture bottle is near atmospheric. One of the plungers is then drawn out approximately 5 mL further before stopcock B is positioned so that all three arms are open. The extra 5 mL in the syringe causes water in the manometer to move up into the pipette, thus ensuring no loss of gas from the immersed end of the pipette. Such a loss would occur if the pressure within the syringes and culture bottle was greater than atmospheric pressure. Final adjustment of the plunger(s) is (are) made to reach atmospheric pressure as indicated by the manometer. The volume of gas in excess of the culture bottle's headspace, is given by the sum of the volumes indicated by the syringes.

At this time gas samples can be removed for compositional analysis. Another syringe is inserted directly through the serum stopper and the sample volume is drawn into this syringe. Before removing the sample syringe, the system is again adjusted to atmospheric pressure with syringe D. Thus, the exact volume of gas drawn into the sample syringe is known. The remaining gas in the measuring syringes (D and E) can then be placed back into the sample bottle simply by closing the side arm of stopcock B and forcing the gas from the syringes into the culture bottle.

This two syringe arrangement also allows convenient wastage of a known volume of gas to relieve excess pressure in the culture bottle. The horizontal syringe is normally used for this purpose. With stopcock C open to both syringes and the culture bottle, and stopcock B positioned so that the arm to the manometer is closed, the desired wastage volume is placed in syringe E. Stopcock B is opened to all three arms and the entire system is adjusted to atmospheric pressure. Then stopcock C is closed entrapping the wastage in syringe E, and the arm to the manometer is closed with stopcock B. The gas from syringe D is injected back into the culture bottle and the culture is removed. Stopcock C is opened to syringe E and the waste gas is discharged through the needle.

Accurate accounts of the total gas removed were kept and included in the cumulative amounts of methane produced. The volume of dry methane in each bottle (prior to sampling) was calculated using the expression:

$$(S+V) \cdot \frac{(P - P_w)}{(T + 273)} \cdot \frac{CH_4}{100} \cdot \frac{273}{760} \quad \text{mL} \qquad (3.3)$$

where S = syringe reading when manometer indicates gas is at atmospheric pressure (mL)

V = volume of headspace in culture bottle (mL)

P = barometric pressure (mm Hg)

T = temperature (°C)

P_w = vapour pressure of water at T (mm Hg)

CH_4 = percent methane in headspace gas (after correction for water vapour)

Aliquots of headspace gas removed for methane analyses were saturated with water vapour. Since the peak areas of these samples were compared with those of standards prepared in bottles without water, corrections for the volume of the water vapour had to be made. The methane concentrations determined by GC analysis (% by volume) were divided by a correction factor calculated by the following expression:

$$1-[(D \times 22.4 \text{ L/mole})/(18 \text{ g/mole} \times 1\,000)] \qquad (3.4)$$

where D is the density of water vapour at saturation expressed in g/m^3 from List (1949).

3.2.4 Statistical Methods

At each time of methane or substrate analyses on batch cultures, the data were analyzed using the method of Dunnett (1955) to determine whether mean concentrations in the test cultures were significantly different from the mean control value ($P<0.05$).

Dunnett's method is similar to other multiple comparison tests (e.g. Scheffé's, Tukey's and Duncan's multiple range test; Steel and Torrie, 1980) but it is designed specifically to compare the means of test treatments with the mean of a control. Dunnett's method produces narrower confidence limits for the comparisons of the differences between the means than do the other multiple comparison tests.

4. EVALUATION OF ANALYTICAL METHODS

Three general analytical methods were used throughout this project and each was evaluated to verify its accuracy. These methods include the analysis of phenolic substrates, the determination of methane concentration in the headspace gas of the anaerobic cultures, and the measurement of the volume of gas generated by the cultures. Each of these procedures is considered separately in the following sections.

4.1 Substrate Analyses

Those alkyl phenolics which are commonly found in industrial wastewaters particularly coal conversion wastewaters were initially chosen as test substrates. These phenolics could readily be analyzed using the GC method of Bartle et al. (1977). The external standard method was used for quantitation and the reliability of the method was evaluated for phenol and p-cresol. Calibration curves were produced by injecting 3 μL aliquots of aqueous solutions of phenol or p-cresol standards. Figure 4.1 shows a typical standard curve resulting from the analysis of eight phenol solutions. Because of the peak tailing, the electronic integrator handled the data acquired from large peaks differently than from small peaks. Thus there was a need to consider two concentration ranges for the standards. Typically four standards were prepared at concentrations less than or equal to approximately 100 mg/L and four or

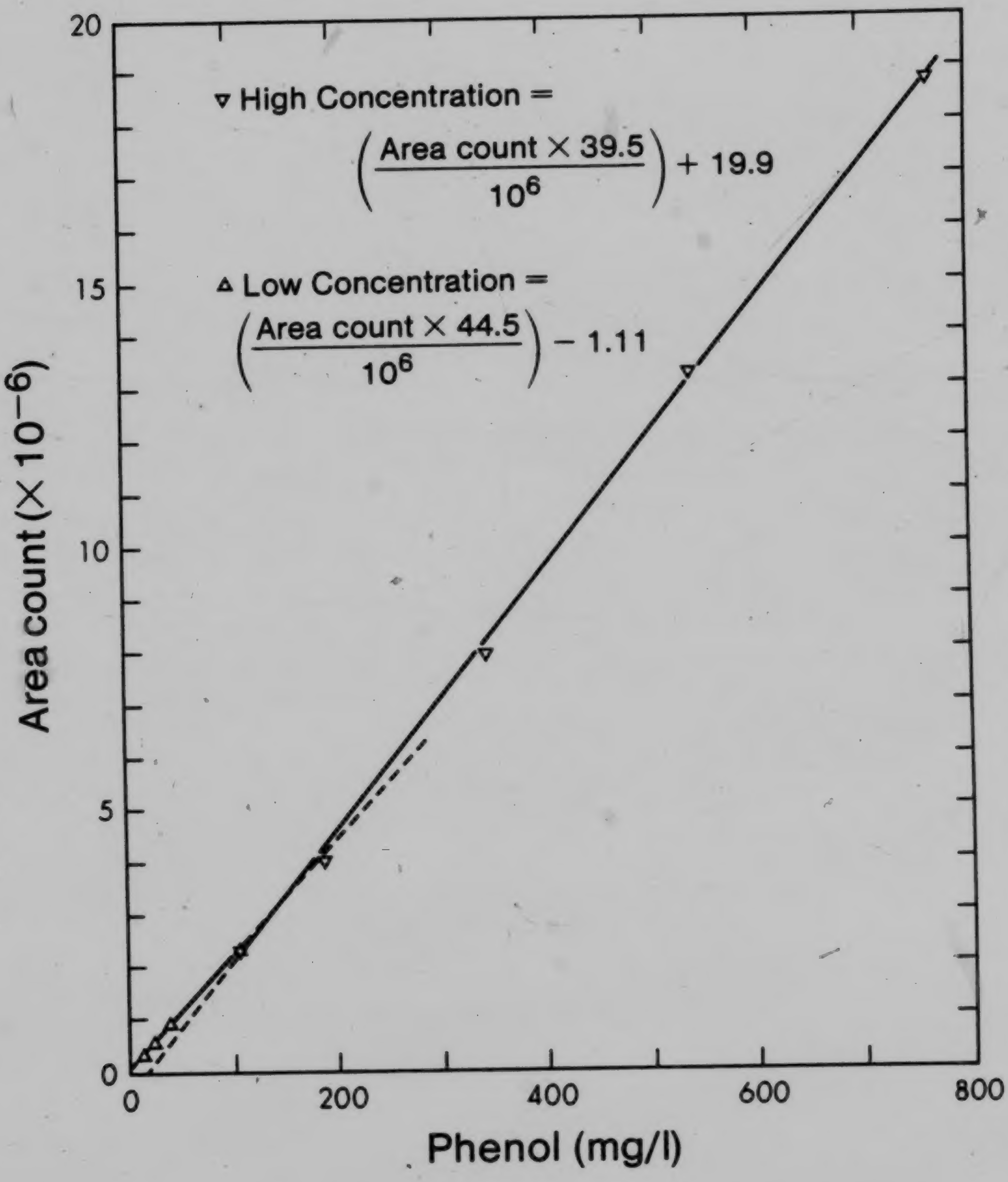


Figure 4.1 Typical standard curves for phenol analysis. The extrapolations of the least squares equations beyond the 100 mg/L standard are shown as broken lines. These illustrate the need for separate sets of high concentration and low concentration standard solutions.

five more were prepared in the range of 100 to 800 mg/L. The area count for the near 100 mg/L standard was considered as part of both the "low-concentration" and the "high-concentration" standard curves when the least squares analysis were applied to the two sets of data. The greatest error was noted when the least squares equation derived from high-concentration standards was applied to a sample which gave a very small area count. Under these conditions, the magnitude of the intercept (19.9 mg/L in Figure 4.1) grossly affected the calculated concentration giving a falsely high result.

Anaerobic sludge samples were spiked with 197 mg/L and 424 mg/L phenol and analyzed in triplicate to determine the accuracy and precision of the method. The mean concentrations and relative standard deviations were found to be 201 mg/L and 0.9%, and 427 mg/L and 2.1%, respectively. Similarly sludge samples were spiked with 138 mg/L and 256 mg/L p-cresol. The mean concentrations and relative standard deviations were found to be 138 mg/L and 0.17%, and 258 mg/L and 2.2%, respectively.

This GC method was very reliable and convenient since it allowed direct sampling of 3 µL aliquots of culture supernatant from the sealed serum bottles. Using the direct aqueous injection technique meant that there was no need for sample preparation and that sampling resulted in a negligible reduction in culture volume.

4.2 Methane Analyses

There were two parameters of concern when determining the validity of the methane analysis. These were the reliability of the standards prepared by the addition of known volumes of methane to a serum bottle filled with air, and the reproducibility of injections into the GC.

The reliability of the standards was tested by preparing gas mixtures between 5 and 40% (V/V) methane in serum bottles. Aliquots of 0.25 mL of each of the eight concentrations were injected into the GC fitted with the thermal conductivity detector and the peak heights measured. The data (shown in Figure 4.2) produced a least squares regression equation (r=0.9993) of:

$$\%CH_4 = [(\text{peak height, in mm})\ \ (0.188)] - 0.038 \qquad (4.1)$$

Two "certified" standard methane in nitrogen mixtures were purchased from Matheson containing 29.9±0.6% and 40.2±0.8% methane, respectively. These gases were analyzed and, based on the regression equation, their mean concentrations ± 2 standard error were found to be 30.8±0.21% and 41.1±0.13%, respectively. The mean values differed from the expected values by only 3.0 and 2.2%, respectively, indicating that the standard preparation method was certainly adequate for these studies.

These data also showed that the thermal conductivity detector gave a linear response up to 40% methane and that only two or three standard mixtures were required to give a calibration curve for any set of culture headspace gas

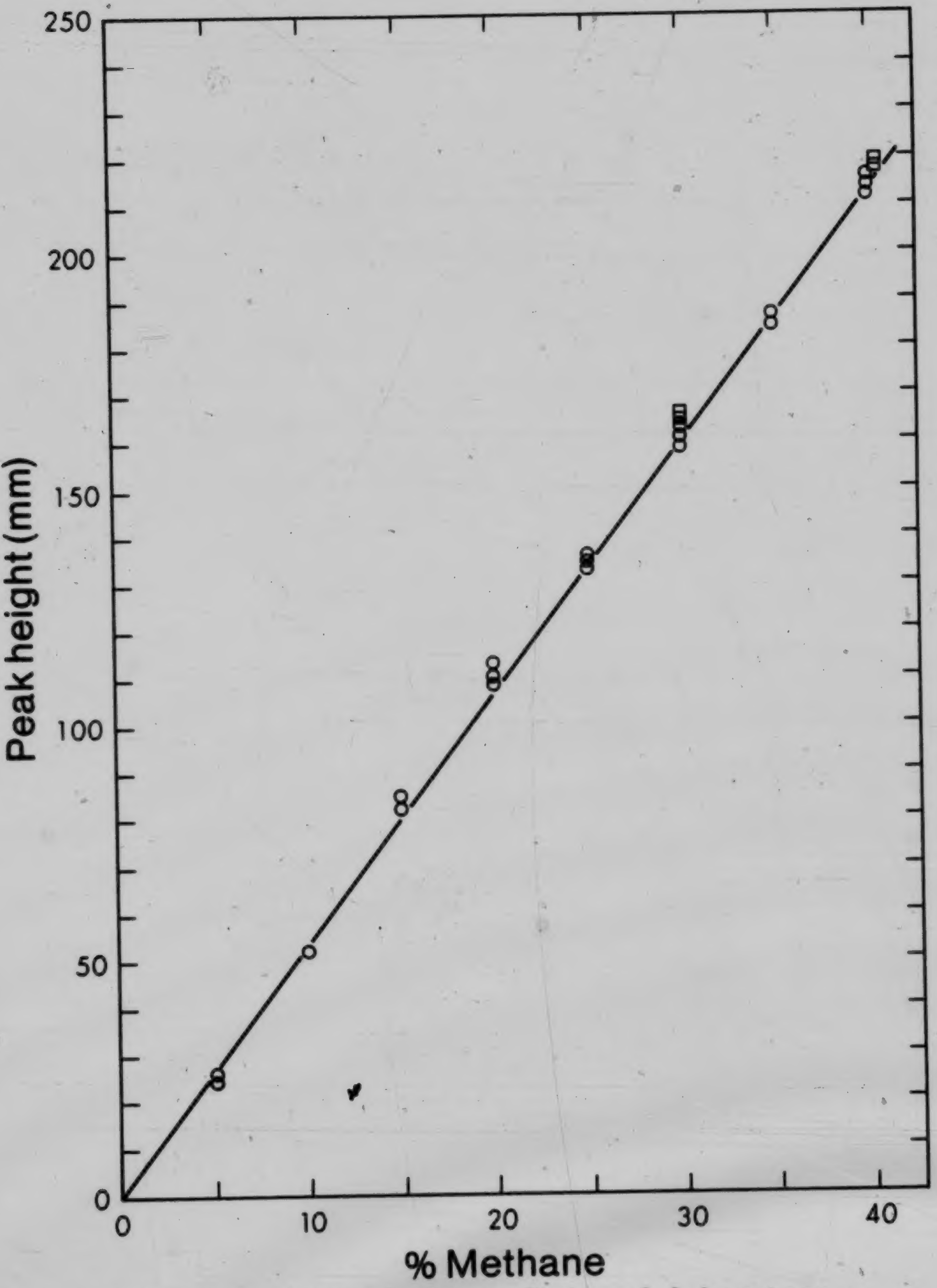


Figure 4.2 Comparison of the analyses of laboratory prepared methane standards (circles) with those of commercially prepared "certified" methane standards (squares).

analyses. This linearity was not always found when the Varian 1700 GC with a flame ionization detector was used for methane quantitation. Thus three to five standard concentrations, which bracketed the sample concentrations, were used to generate the calibration curve to minimize the analysis error.

The three types of syringes considered for gas sampling during this study were the 1 mL B-D Glaspak Tuberculin syringe, the 100 µL Hamilton gas-tight syringe (1710-TEFLL) with a luer-lok end, and the 0.5 mL B-D Plastipak Lo-Dose syringe. Of the three types evaluated, the expensive Hamilton syringe gave the least reproducible results. The disposible hypodermic needles used with this syringe (27G x 12 mm) were found to have a dead volume of approximately 38 µL when fitted onto the syringe. This meant that up to a 38% variation in sampling could be expected depending upon the number of times that the syringe was flushed with the culture headspace gas. The flame ionization detector was more sensitive to non-reproducible sampling and the 0.5 mL Lo-Dose syringe was found to be the most reliable. When the GC with the less sensitive, thermal conductivity detector was used, both the Glaspak and the Lo-Dose syringes gave adequately reproducible results.

The Lo-Dose syringe has a fixed needle with essentially zero dead volume and a barrel that is calibrated so that gas samples as small as 0.1 mL can be reproducibly removed from the serum bottles. When eight standard methane mixtures (between 1 and 24%) were prepared and five, 0.2 mL aliquots from each were analyzed using the Lo-Dose syringe, the percent relative standard deviations of the peak heights were found to range from 1.7 to 3.6%

4.3 Gas Volume Measurements

The validity of the gas measurements made with the manometer assembly shown in Figure 3.1, was proven by the following series of tests and observations.

Early experience with the apparatus described by Boone (1982) showed that the longer the culture bottle was held during the measurement procedure, the greater the measured gas volume. That is, the culture bottle was acting as a gas thermometer. Thus a method of insulating the bottle from the experimenter's hand was incorporated into the procedure. The effectiveness of the insulator was proven by the following experiment.

Two sets of serum bottles were sealed and a hypodermic needle was inserted through the stopper of each bottle. These were allowed to sit for approximately 1.5 h to reach ambient temperature and pressure and then the needles were removed. Using a Hamilton 10 mL gas-tight syringe, a co-worker added various amounts of air to the serum bottles and the experimenter then measured the air in the bottle to determine how closely the measurements agreed with the actual amounts of air added. One set of bottles was measured without the use of the insulator while the second set was measured using the insulator.

Table 4.1 shows the results of gas volume measurements with and without the insulator. In each case without the insulator, the measured volume was greater than the volume of air added. With the insulator, the differences ranged from -0.2 to +0.1 mL with a mean difference near zero. Thus the insulator removes a source of error which produced falsely high results.

To further evaluate the apparatus, sealed serum bottles were equilibrated to ambient temperature and pressure as outlined above. Each bottle was then tared and glycerol was injected through the stopper from a glass syringe fitted with an 18G x 3.8 cm needle. The actual volume of glycerol added was calculated by dividing the weight of glycerol in the bottle by its density (1.26 g/mL). After sitting at ambient temperature for 1 h, the volume of air displaced by the glycerol was determined using the gas measuring apparatus.

Glycerol was used to displace air in sealed serum bottles because its vapour pressure at room temperature is less than 1 mm Hg and therefore no correction for this parameter needed to be applied. The results of 14 gas measurements with volumes of glycerol up to 30 mL are plotted against the expected volumes for each test case (Figure 4.3). All measurements were made with the 30 mL syringe. The maximum difference between the measured and the expected volumes was 0.37 mL in the bottle that contained

Table 4.1 Comparison of gas volume measurements made with and without the insulating sample bottle holder.

Without Insulator			With Insulator		
Air added (mL)	Volume measured (mL)(1)	Difference (mL)	Air added (mL)	Volume measured (mL)(1)	Difference (mL)
1.0	1.3	+0.3	4.0	3.8	-0.2
2.0	2.3	+0.3	2.0	2.0	0
3.0	3.4	+0.4	6.0	6.1	+0.1
4.0	4.2	+0.2	3.0	3.1	+0.1
5.0	5.4	+0.4	5.0	5.1	+0.1
6.0	6.4	+0.4	7.0	7.0	0
			1.0	0.8(2)	-0.2
	mean = +0.3			mean = -0.01	

(1) With 10-mL syringe attached to the manometer.

(2) When a 1-mL syringe was attached to the manometer, the volume measured was 0.96 mL.

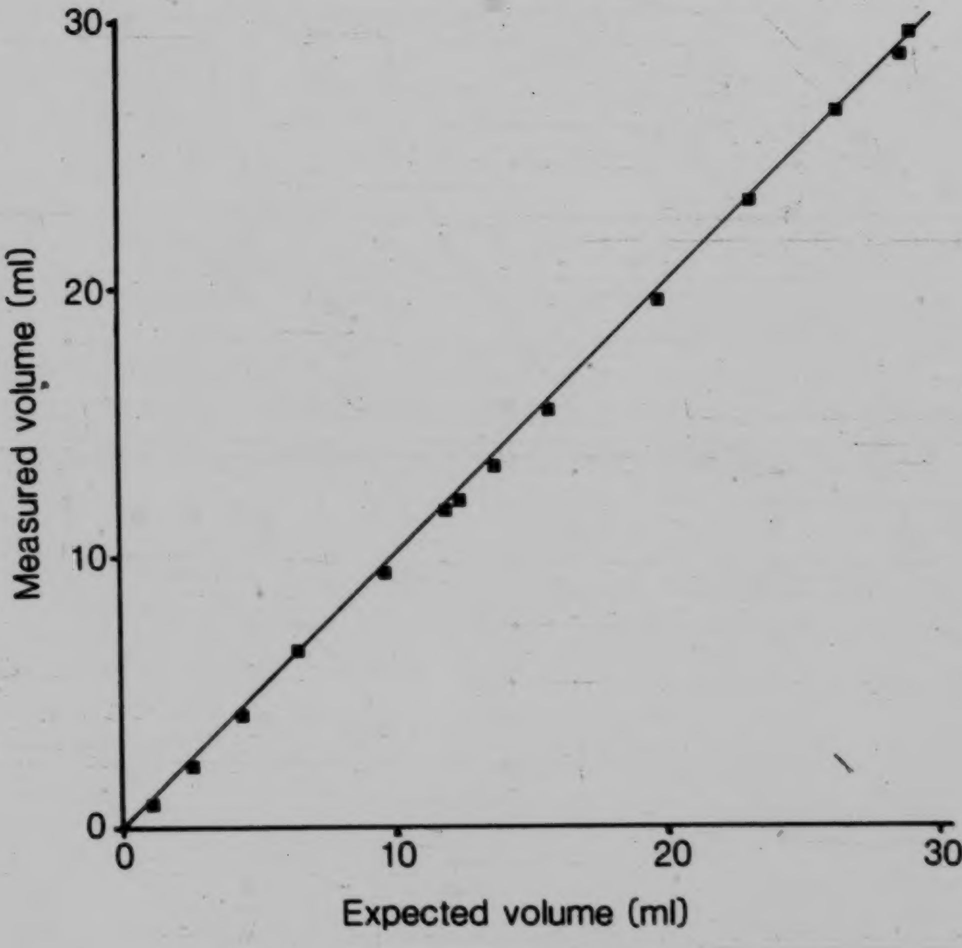


Figure 4.3 Measured volumes of air displaced by known amounts of glycerol added to sealed serum bottles. (Solid line gives expected values.)

28.87 mL glycerol. The relative deviations from the expected values decreased as the volume of glycerol added increased. In eight of the nine cases where the expected volume was greater than 10 mL, the deviations were less than 2% while in the ninth case it was 3.5%.

In another test, 15.0 mL aliquots (at ambient temperature and pressure) of methane (Matheson CP) were added to six sealed 158 mL serum bottles which had been equilibrated to ambient temperature and pressure (24°C, 757 mm Hg). The apparatus was then used to measure the volume of gas in each bottle and during this procedure three aliquots (0.1 mL each) were removed for GC analysis to determine the percent methane in each bottle. The gas remaining in the measuring syringe (D) (Figure 3.1) was then compressed back into the bottle.

To mimic culture conditions, 10 mL aliquots of water were then added to each of these serum bottles. After vigourous shaking and a 1-h equilibration period the gas volume and percent methane in the headspace gas were again determined.

The data in Table 4.2 illustrate the accuracy and precision of the apparatus for quantitative measurement of methane in serum bottles. The volume of methane added to the serum bottles was expected to give a concentration of 8.67% and the observed values were between 8.5% and 8.8%. When corrected to standard conditions of temperature and pressure (STP), 13.73 mL of methane should have been found. The sum

Table 4.2 Quantitative measurement of a known amount of methane in sealed serum bottles.

Dry bottles				10 mL water in each bottle			
	Methane				Methane		
Gas Volume measured (mL)[1]	Percent by volume[2]	Total Volume in bottle (mL)[3]	Recovery (%)	Gas volume measured (mL)[1]	Percent by volume[2]	Total volume in bottle (mL)[3]	Recovery (%)
14.7	8.7	13.8	101	27.9	8.7	13.7	100
14.5	8.7	13.7	100	27.9	8.7	13.7	100
14.3	8.6	13.6	99	25.5	8.5	13.2	96
14.6	8.7	13.7	100	28.0	8.8	13.8	101
14.4	8.6	13.6	99	28.0	8.6	13.5	99
14.6	8.7	13.7	100	27.9	8.8	13.8	101
			mean = 99.8				mean = 99.5

[1] syringe reading

[2] based on GC analyses

[3] dry gas at STP

of the volume of gas measured plus the headspace volume in the bottle was multiplied by the methane concentration to give the total volume of methane at ambient conditions. These were then adjusted to STP with the results shown in column 4 of Table 4.2. The recoveries observed in the dry bottles ranged from 99% to 101% with a mean of 99.8%.

The addition of 10 mL water to these bottles increased the gas volume measurements by approximately 13 mL over those observed with the dry bottles. This was due to the displacement of gas by the water and to the saturation of the gas phase with water vapour. The expected amount of methane was 13.71 mL (at STP), slightly less than in the dry bottles since three aliquots of 0.1 mL each had been removed for GC analyses. Again the recoveries were excellent ranging from 96% to 101% with a mean of 99.5%.

When using this apparatus, or any of the others described in the literature, there is always a slight volume of gas that remains in the dead volume of the device, at a pressure greater than atmospheric, when the gas is compressed back into the culture bottle. When the bottle is removed, this gas is lost. With this in mind, the apparatus was constructed to have a minimum dead volume. The first version had a "T" connector in place of stopcock B and a pinch clamp on the tubing. However, in the present apparatus, stopcock B has a smaller dead volume than the "T" connector and it also excludes the volume of the Tygon tubing, between the "T" connector and the pinch clamp, from the overall dead volume. The magnitude of this error is small and is not apparent in the data given in Table 4.2 where the measured gas was placed back into the dry bottles prior to the addition of water.

This entire measuring procedure takes less than 2 min per culture bottle and provides an accurate record of the amount of gas produced, wasted and removed for compositional analysis. This apparatus has proven to be reliable and easy to use and maintain.

5. FERMENTABILITY AND EFFECTS OF INDIVIDUAL PHENOLICS IN BATCH CULTURES

The initial experiments in this project were designed to determine which alkyl phenolics could be degraded anaerobically to yield methane. Since these compounds are toxic to microorganisms, a variety of concentrations of each was tested to determine their effects on methanogenic batch cultures. The activities of these cultures were monitored by frequent methane analyses of the headspace gas. The amount of methane evolved was compared to that of control cultures which received no phenolic substrate. The addition of a particular phenolic concentration that gave more methane production than the control indicated that the compound was fermentable. If the methane production was significantly lower than the control, the test phenolic concentration was clearly inhibitory to the anaerobic fermentation.

The effects of the alkyl phenolics on the latter stages of the anaerobic process - that is the conversion of volatile organic acids (VOAs) to methane - were tested in batch cultures which were supplemented with acetate and propionate. In these cultures, acetate was available to the methanogens immediately upon inoculation. Thus any direct inhibition of these organisms was indicated by reduced methane concentrations when compared to those of the control cultures.

During this phase of the study, only two of the phenolics - phenol and p-cresol - were found to be fermentable although data presented in chapter 7 show that m-cresol can also be degraded anaerobically. Batch cultures containing accurately known amounts of either phenol or p-cresol were established and the ultimate amounts of methane produced per unit mass of substrate were determined. These values were required for later work with semicontinuous cultures which were individually fed one of these substrates.

For most of the experiments in this project, domestic anaerobic sludge was used as an inoculum. However, three sludges from other sources were obtained and tested with a number of phenolics and related compounds to determine whether the microorganisms in these sludges were able to degrade a wider variety of compounds than those organisms in the domestic sludge. The three new sources of inocula came from anaerobic environments in which phenolics were present.

5.1 Procedures

5.1.1 Initial Screening Experiments

Ten phenolics were individually tested for their fermentability and inhibitory concentrations. These included phenol, the three isomers of cresol (methylphenol) and the six isomers of dimethylphenol. The final concentrations of each phenolic in the inoculate cultures were as follows: 500, 1 000, 2 000 mg/L phenol; 200, 400, 1 000 mg/L cresol; or 100, 300, 500 mg/L dimethylphenol.

Solutions of phenolics were prepared in distilled water at concentrations ten times greater than their desired concentrations in the test cultures. A 1 mL aliquot of phenolic solution was placed into a 68 mL serum bottle and O_2-free 30% CO_2 in N_2 was bubbled through the aliquot for 2 to 3 min at approximately 100 mL/min. Four mL of medium, (section 3.1) were added to the bottles which were then sealed and autoclaved. Three bottles of each substrate concentration were used. Control cultures were prepared in the same manner with 1 mL distilled water substituted for the phenolic solution.

Immediately prior to use, 0.1 mL sulfide solution was injected into each bottle. Using a syringe fitted with a 21G x 2.5 cm needle these were inoculated with 5 mL sludge from the anaerobic digesters at the Edmonton Gold Bar Wastewater Treatment Plant. All cultures were incubated at 37°C for a minimum of 40 days and samples of the headspace gas were analyzed for methane.

5.1.2 Cultures Supplemented With Fermentable Substrates

The o- and m- isomers of cresol and the six isomers of dimethylphenol were tested to determine their inhibitory effect on conversion of VOAs to methane. Each of these phenolics was tested at the two highest concentrations listed in section 5.1.1. Prior to autoclaving, each culture bottle received 0.1 mL of a solution containing 37.5 mg/mL acetic acid and 13 mg/mL propionic acid (adjusted to pH 7 with NaOH).

The effects of various concentrations of fermentable phenolics (phenol and p-cresol) were further studied in batch cultures. Three types of medium were used in these experiments. These included unsupplemented medium (Table 3.2); the acetic acid and propionic acid supplemented medium described above; and a medium which contained nutrient broth and glucose. The last experiment used 3 mL of concentrated growth medium (section 3.2.1) to which 1 mL of a solution (designated NBG) containing 2 000 mg/L nutrient broth (Difco) and 2 000 mg/L glucose were added. In all cases 1 mL of phenolic solution was added prior to autoclaving to give the following final concentrations after inoculating with 5 mL of domestic anaerobic sludge: 0, 500, 800, 1 000, and 1 200 mg/L phenol or 0, 200, 400, and 600 mg/L p-cresol. Methane production was monitored over the incubation periods which ranged from 50 to 70 days.

5.1.3 Substrate Loss From Methane Producing Cultures

To verify that enhanced methane concentrations in batch cultures containing fermentable phenolics were the result of degradation of these substrates three batch cultures were established. One contained 450 mg/L phenol; another 160 mg/L p-cresol and the third contained no added phenolics and served as a control culture. These were incubated at 37°C and methane production and substrate concentrations were monitored using the appropriate GC methods.

5.1.4 Fermentable Substrate Concentration and Acclimation Time

The effects of fermentable phenolic substrate concentration on batch culture acclimation time were evaluated by monitoring methane production from cultures containing: 0, 100, 200, 300, 400, and 500 mg/L phenol or 0, 100, 200, 300 and 400 mg/L p-cresol.

5.1.5 Ultimate Gas Production from Phenol and p-Cresol

A solution containing 399.0 mg phenol was prepared in 100 mL in a volumetric flask. Aliquots of 5.0 mL of this solution were pipetted into three 158 mL serum bottles containing 0.5 mL resazurin solution. After approximately 3 min of gassing these liquids with O_2-free 30% CO_2 in N_2, 45 mL of domestic anaerobic sludge were added to each bottle which was then immediately sealed. Similarly three bottles containing 5.0 mL distilled water, rather than phenol solution, were prepared to serve as controls.

These bottles were allowed to sit at room temperature for 1 h and then the pressure within the bottle was adjusted to atmospheric. This was done by placing approximately 10 mL O_2-free 30% CO_2 in N_2 in the gas measuring apparatus (Figure 3.1) and inserting its needle into a culture bottle. If the manometer indicated that the pressure within the bottle was less than atmospheric, gas from the measuring device was added to adjust the pressure. If the pressure within was greater than atmospheric, gas was removed using the syringe of the measuring device. Each control bottle was randomly paired with a phenol containing bottle. After various times of incubation, the volume of dry methane at STP in each bottle was determined. The net amount of methane produced by each phenol-containing culture was the differences between the methane in the test and control cultures in the paired set.

Similarly cultures were established using 5.0 mL aliquots of a p-cresol solution (298.9 mg/100 mL) to determine the ultimate gas production from this substrate.

5.1.6 The Ability of Alternate Sources of Inocula to Degrade Phenolics

Three anaerobic sludges which had been exposed to a variety of phenolic compounds were obtained and these were used as inocula to test the fermentability of a wide number of phenolics. The compounds and their tested concentration are summarized in Table 5.1. For each substrate, methane concentrations were measured and compared to controls which received 1 mL distilled water in place of the substrate solution.

The first sludge was obtained from the 15 m depth of a tar sands tailings pond and was used for two batch experiments. In the first trial, 4 mL medium and 1 mL substrate solution were inoculated with 3 mL of the tailings pond sludge and 2 mL of domestic anaerobic sludge. One set of cultures containing glucose and one containing VOAs were

Table 5.1 Substrate concentrations (mg/L) used in fermentability studies with various inocula from phenolic-containing enviroments.

Substrate	Oilsands tailing pond sludge		Coking plant sludge	Coal conversion sludge
	Trial 1	Trial 2		
glucose	600	NT(1)	300	300
VOAs(2)	505	NT	505	505
phenol	400	200	200	200
p-cresol	200	200	200	200
m-cresol	200	200	200	200
o-cresol	200	200	200	200
2,5-dimethylphenol	100	100	100	100
2,6-dimethylphenol	100	100	100	100
3,4-dimethylphenol	100	100	100	100
3,5-dimethylphenol	100	100	100	100
p-ethylphenol	200	200	200	200
2,3-dihydroxytoluene	NT	200	200	200
2,4-dihydroxytoluene	200	200	200	200
1-naphthol	NT	100	100	100
2-naphthol	NT	100	100	100
catechol	NT	NT	NT	200
resorcinol	NT	NT	NT	200
industrial wastewater	NT	NT	10(3)	4(3)

(1) NT = not tested

(2) 375 mg/L acetic acid and 130 mg/L propionic acid

(3) Percent by volume

established as positive controls to test that no inhibition of the anaerobic process was caused by the tailings pond sludge. Ten phenolics were tested in the first trial while 13 were tested in the second trial. In the latter case, the sizes of the inocula were increased to 9 mL of the tailings pond sludge and 4 mL of the domestic anaerobic sludge. These were added to 14 mL medium plus 1 mL phenolic solution.

Two sludge samples were supplied by Dearborn Environmental Consulting Services, Mississauga Ontario. The first sludge came from a laboratory scale anaerobic filter treating 80% strength ammonia stripped wastewater from a coal coking operation near Pittsburgh, Pennsylvania. Cultures fed glucose, VOAs and the ammonia stripped wastewater served as positive controls to ensure that the sludge was still active after transport. Since the inoculum came from a methane producing reactor, no domestic anaerobic sludge was added to the culture bottles which contained 4 mL medium, 1 mL phenolic solution and 5 mL inoculum. Thirteen phenolics were tested as shown in Table 5.1.

The second sludge was taken from a pilot scale fluidized bed anaerobic filter being operated at the Wastewater Technology Centre in Burlington, Ontario. This reactor was being acclimated to treat a coal conversion wastewater from a pilot scale H-coal process in Catlettsburg, Kentucky. Before being fed to the anaerobic filter, the ammonia-stripped H-coal effluent had been extracted with methylisobutylketone and mixed with four parts wastewater from a starch processing plant.

Fifteen individual phenolic compounds (Table 5.1) were tested in batch cultures containing 4 mL medium, 1 mL phenolic solution and a 5 mL inoculum of sludge #2 which was known to have an active methane producing population. Five positive control cultures were established containing one of the following: glucose, VOAs, phenol, p-cresol, and 4% (V/V) H-coal effluent which had not been methylisobutylketone extracted. This wastewater was thought to contain catechol and resorcinol (Dearborn Environmental Consulting, personal communication) and therefore these dihydroxybenzenes were also tested.

5.2 RESULTS AND DISCUSSION

5.2.1 Initial Screening Experiments

The experiments described here were designed to indicate whether methane production was inhibited, unaltered or enhanced by the phenolic concentration within the culture. Thus, measurement of the methane concentration in the headspace gas, expressed as percent by volume (not corrected for temperature and pressure), was appropriate since all results were compared to control cultures at the same conditions. Each culture bottle contained fermentable organics which were present in the filtered sludge (as part of the medium) and in the 5 mL sludge inoculum. Therefore, inhibition of the cultures by any of the phenolics would result in less methane in the test cultures than in the control cultures (Owen et al., 1979).

During their screening experiments, Chmielowski et al. (1965) found catechol, hydroquinone, o- and m-cresol, 2,4-, 3,5-, and 3,4-dimethylphenol to be resistant to anaerobic fermentation. However, since Healy and Young (1978) and Balba and Evans (1980a) have demonstrated that catechol can be fermented to methane, and Chou et al. (1978) have shown that hydroquinone can be degraded anaerobically, the fermentability of some of the alkyl phenolics tested by Chmielowski et al. (1965) was reevaluated.

The results of screening phenol and nine alkyl phenolics at various concentrations are shown in Table 5.2. None of the six isomers of dimethylphenol inhibited nor stimulated methane production at a concentration of 100 mg/L. At 300 mg/L, three of the dimethylphenol isomers (2,3-, 2,4-, and 3,4-) showed slight inhibition of the fermentation. In the worst case (2,3-) there was 17% less methane in the test cultures than in the control cultures. All but one isomer of dimethylphenol (2,6-) reduced the amount of methane produced in these batch cultures when they were present at 500 mg/L. However, the methane production was only decreased by 30% to 40% of that in the controls. The dimethylphenol fermentability results, including those for the 2,3-, 2,5-, and 2,6- isomers which had not previously been tested by Chmielowski et al. (1965), confirmed their resistance to methanogenesis. As well,

Table 5.2 Summary of inital screening experiments. Batch cultures incubated for 42 to 49 days.

Substrate	Concentration (mg/L)	Amount of methane at end of incubation period as compared to control cultures (%)
2,3-dimethylphenol	100	94
	300	83•
	500	60•
2,4-dimethylphenol	100	97
	300	86•
	500	67•
2,5-dimethylphenol	100	100
	300	94
	500	74•
2,6-dimethylphenol	100	108
	300	102
	500	94
3,4-dimethylphenol	100	100
	300	91•
	500	69•
3,5-dimethylphenol	100	99
	300	89
	500	67•
o-cresol	200	111
	400	118•(1)
	1 000	67•
m-cresol	200	100
	400	95
	1 000	55•
p-cresol	200	162•
	400	102
	1 000	63•
phenol	500	203•
	1 000	93
	2 000	44•

(1) This enhanced methane concentration was not observed in later attempts to ferment o-cresol.

• Significantly different from controls ($P<0.05$).

levels up to 500 mg/L were not extremely inhibitory to the fermentation of other degradable substrates in the sludge.

o-Cresol at 200 mg/L was not fermented nor did its presence inhibit methane formation from degradable substrates in the medium (Table 5.2). Similar results were found with m-cresol at 200 and 400 mg/L. The apparent enhanced methane production observed in the cultures containing 400 mg/L o-cresol during the initial screening experiments could not be reproduced in two other experiments. Thus it is unlikely that o-cresol is fermentable and it is certainly not inhibitory at this concentration. However, inhibition was observed when the concentration of each cresol reached 1 000 mg/L. Methane generation was reduced to 55% to 67% of the control values at the end of the 42-day incubation period.

When present at 200 mg/L, p-cresol gave enhanced methane production (Table 5.2) indicating that it was fermented to methane as has been demonstrated by others (Chmielowski et al., 1965; Balba et al., 1981). Similarly, phenol at 500 mg/L was fermented agreeing with reports of Chmielowski et al., (1965) and Healy and Young (1978). As the concentrations of these two fermentable phenolic substrates were increased, (400 mg/L p-cresol and 1 000 mg/L phenol) elevated methane concentrations were no longer observed, but the methane productions paralleled those of the control cultures. At higher concentrations (1 000 mg/L p-cresol and 2 000 mg/L phenol) inhibition of the anaerobic process was indicated by both a decreased rate of methane production and a lower methane concentration at the end of the 42-day incubation period.

5.2.2 Cultures Supplemented With Fermentable Substrates

The observed reduction of methane production in cultures containing high concentrations of phenolics could be due to:

a. inhibition of the methane bacteria;
b. inhibition of the non-phenolic-degrading acid-formers, thus reducing the VOAs available for the methane bacteria; or
c. a combination of these two factors.

The effects of phenolic concentrations on the conversions of VOAs to methane can be determined by adding acetate and propionate to the medium and monitoring methane production (Owen *et al.*, 1979). In this way, during the early stages of incubation, the methanogens are not dependent upon the non-methanogens for their source of substrate.

For each of the eight non-fermentable phenolics, VOA-supplemented cultures were set up with the individual phenolic concentrations at the two highest levels shown in Table 5.2. The average concentrations of methane measured on day 1 and on the final day of incubation (day 41 to 43) were compared to control cultures which contained no added phenolic. These data are summarized in Table 5.3. An immediate inhibition was observed on day 1 with seven of the

Table 5.3 Effects of some alkyl phenolics on the conversion of VOAs to methane.

Substrate	Concentration (mg/L)	Amount of methane as compared to control (%)	
		Day 1	End of incubation
2,3-dimethylphenol⁽¹⁾	300	87*	92*
	500	40*	58*
2,4-dimethylphenol⁽¹⁾	300	80*	88*
	500	38*	56*
2,5-dimethylphenol⁽²⁾	300	82	93
	500	26*	75*
2,6-dimethylphenol⁽²⁾	300	111	99
	500	99	91*
3,4-dimethylphenol⁽²⁾	300	99	95
	500	37*	63*
3,5-dimethylphenol⁽²⁾	300	73*	92
	500	20*	57*
o-cresol⁽³⁾	400	67*	98
	1 000	35*	20*
m-cresol⁽³⁾	400	78*	100
	1 000	28*	14*

(1) Incubation time = 43 days.

(2) Incubation time = 42 days.

(3) Incubation time = 41 days.

* Significantly lower than the controls ($P<0.05$).

eight phenolics at the higher concentration tested. The exception was 2,6-dimethylphenol. The 60% to 80% reductions in methane production indicated that the methane bacteria were being directly affected by the presence of the phenolics. The analyses on day 1 showed that the lower concentrations were less inhibitory. In the cases of 2,5-, 2,6-, and 3,4-dimethylphenols, the methane levels were not significantly lower than controls.

By the end of the incubation periods, the relative amounts of methane produced in the batch cultures containing dimethylphenols had increased in all but one case (the 2,6-isomer). However, at 500 mg/L all six isomers showed significantly less methane than did the controls. At 300 mg/L, only the 2,3- and 2,4- isomers gave significantly lower methane levels than the control cultures.

Figure 5.1 shows the methane production from the VOA-supplemented cultures which received increased concentrations of 2,3-dimethylphenol. This plot is typical of most of those cultures tested with a dimethylphenol and illustrates the immediate inhibition of methane when the 500 mg/L of the compound was present.

Methane production in VOA-supplemented cultures containing 400 mg/L o- or m-cresol was slightly inhibited during the initial period of incubation (Table 5.3) but these recovered by day 41, when the methane concentrations did not differ from those in the controls. However at 1 000 mg/L, recovery did not occur and the methane

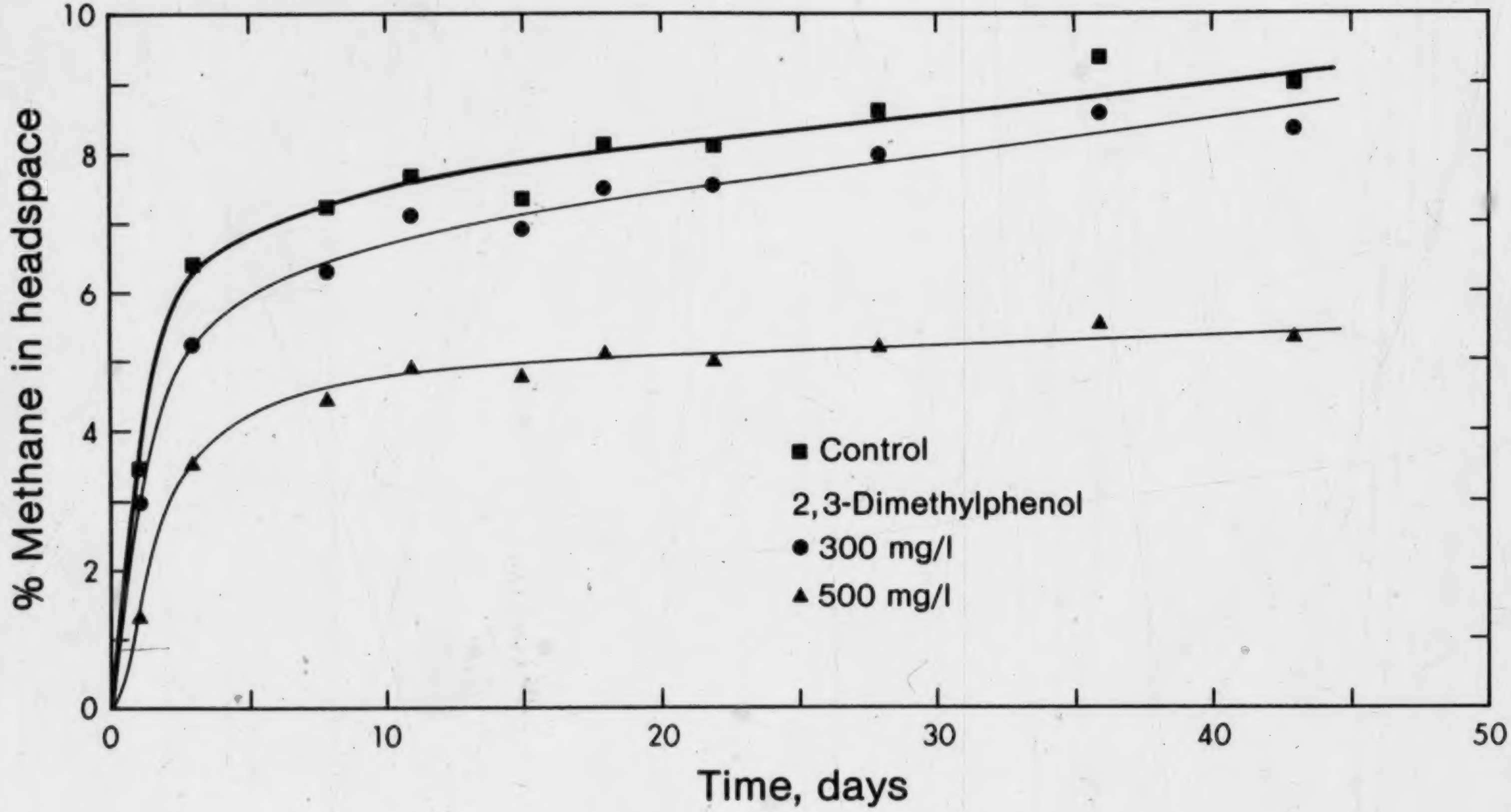


Figure 5.1 Methane production in batch cultures containing various concentrations of 2,3-dimethylphenol - with VOA supplementation.

concentrations in the o-cresol and m-cresol cultures were only 20 and 14%, respectively, of the control values.

Based on the responses of batch cultures to increasing concentrations of the fermentable phenolics (i.e. phenol and p-cresol) (Table 5.2) there appear to be three distinct microbial populations in the inoculum as shown in Figure 5.2. Within the non-methanogenic phase there are acid-formers which cannot degrade the phenolic substrates (designated non-phenolic-degrading acid-formers) and those which can degrade these substrates (designated phenolic-degrading acid-formers). In addition to these two groups, there are, of course, the methane bacteria. Further studies with batch cultures containing a wider range of phenol and p-cresol concentrations in unsupplemented, VOA-supplemented and NBG-supplemented media were done to demonstrate the effect of phenolic concentrations on the three populations.

Figure 5.3 summarizes the methane production from non-VOA-supplemented cultures which received varying amounts of phenol. After an acclimation time of about 15 days, those containing 500 mg/L phenol produced significantly more methane than did the controls. At concentrations of 800, 1 000, and 1 200 mg/L, none of the mean methane concentrations were significantly different ($P<0.05$) from that of the control cultures. (The 800 and 1 000 mg/L data are not shown in Fig 5.3 to avoid congestion.) These results indicate that the phenol-degrading acid-formers (Figure 5.2) are susceptible to inhibition at phenol concentrations

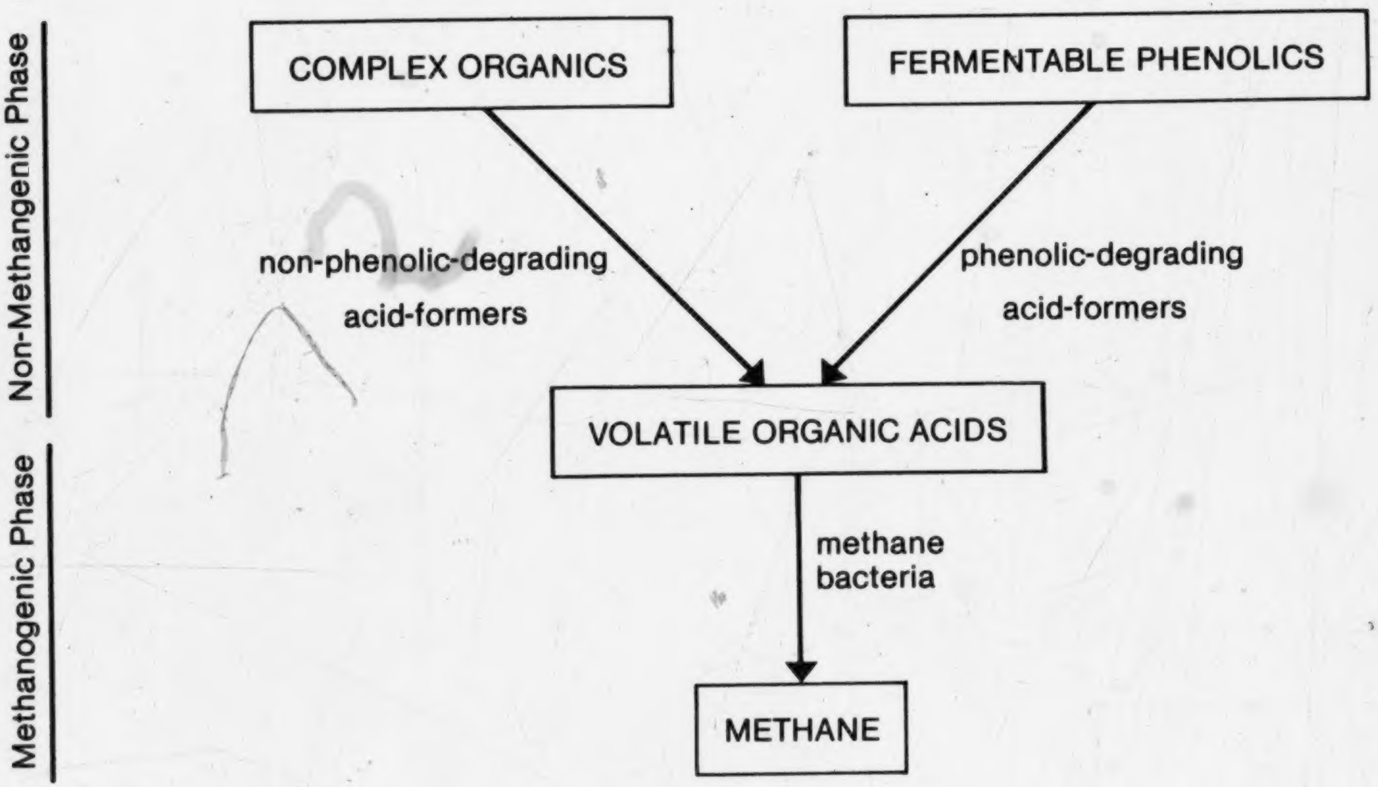

Non-Methangenic Phase
Methanogenic Phase
COMPLEX ORGANICS
FERMENTABLE PHENOLICS
non-phenolic-degrading acid-formers
phenolic-degrading acid-formers
VOLATILE ORGANIC ACIDS
methane bacteria
METHANE

Figure 5.2 Microbial activities leading to methane production in the presence of fermentable phenolics.

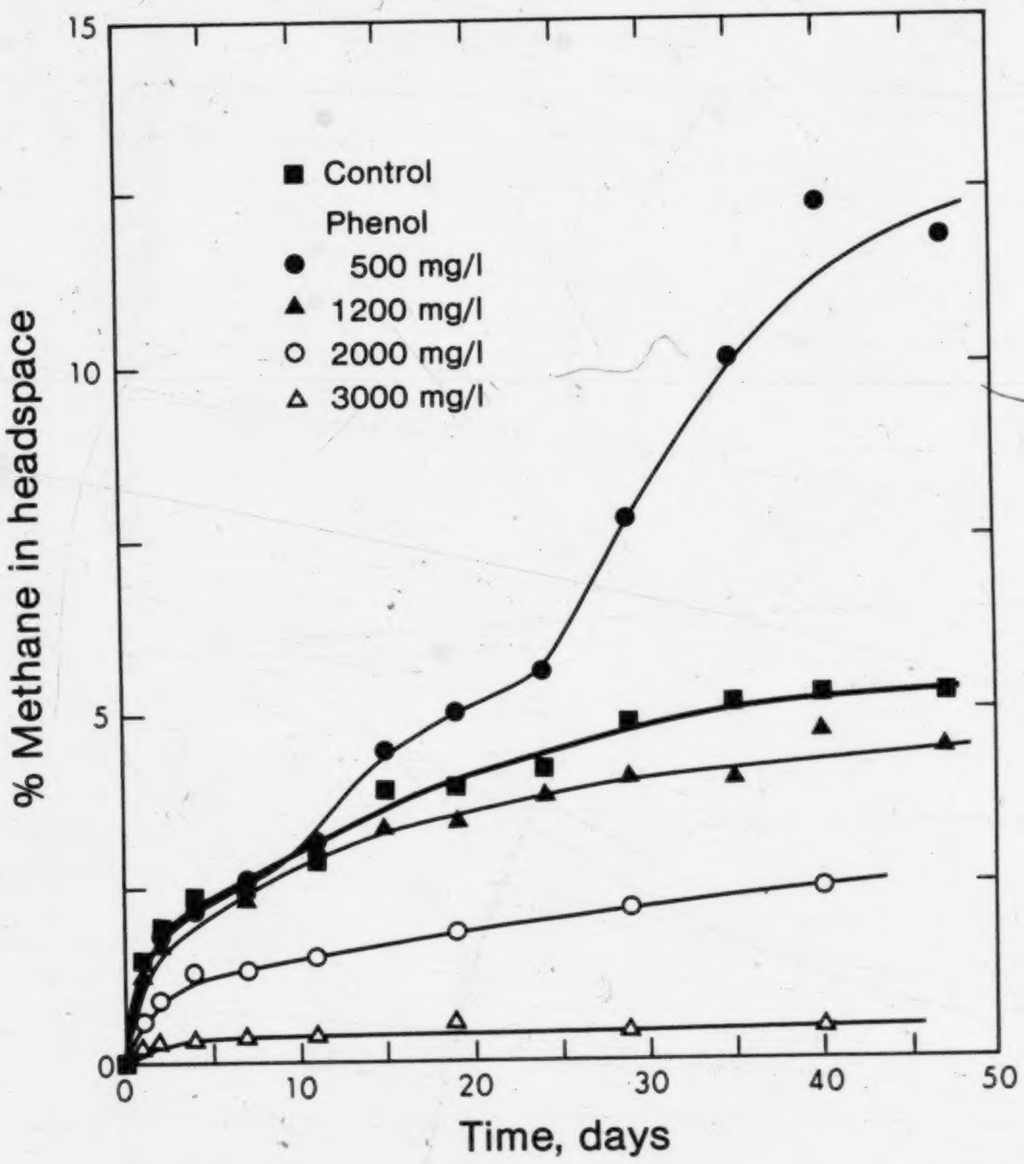


Figure 5.3 Methane production in batch cultures containing various concentrations of phenol - without VOA supplementation.

between 800 and 1 200 mg/L, but neither the non-phenol-degrading acid-formers nor the methanogens are inhibited. As the phenol concentration was increased to 2 000 and 3 000 mg/L, the concentrations of methane produced decreased. Therefore, at these substrate concentrations, either the non-phenol-degrading acid-formers or the methane bacteria, or both groups are being inhibited.

In the case of the VOA-supplemented cultures (Figure 5.4) there was a much faster initial methane production rate, relative to the unsupplemented cultures (Figure 5.3), at phenol concentrations less than or equal to 1 200 mg/L. For example, the day 4 value was about 5% methane (in the headspace gas) for the supplemented control cultures as compared to 2.5% methane with the unsupplemented controls. The cultures containing 500 mg/L phenol produced significantly more methane than the controls after 29 days incubation. As observed with the non-supplemented cultures, phenol concentrations of 800, 1 000, and 1 200 mg/L did not significantly ($P<0.05$) stimulate nor inhibit methane production. However, concentrations of 2 000 and 3 000 mg/L phenol did inhibit methane formation. Since acetate was added to these cultures, the methane bacteria (Figure 5.2) were being inhibited at these concentrations. Whether these elevated phenol concentrations are also inhibitory to the non-phenol-degrading acid-formers has not been determined.

The stimulation and inhibition trends observed with varying phenol concentrations were also observed with

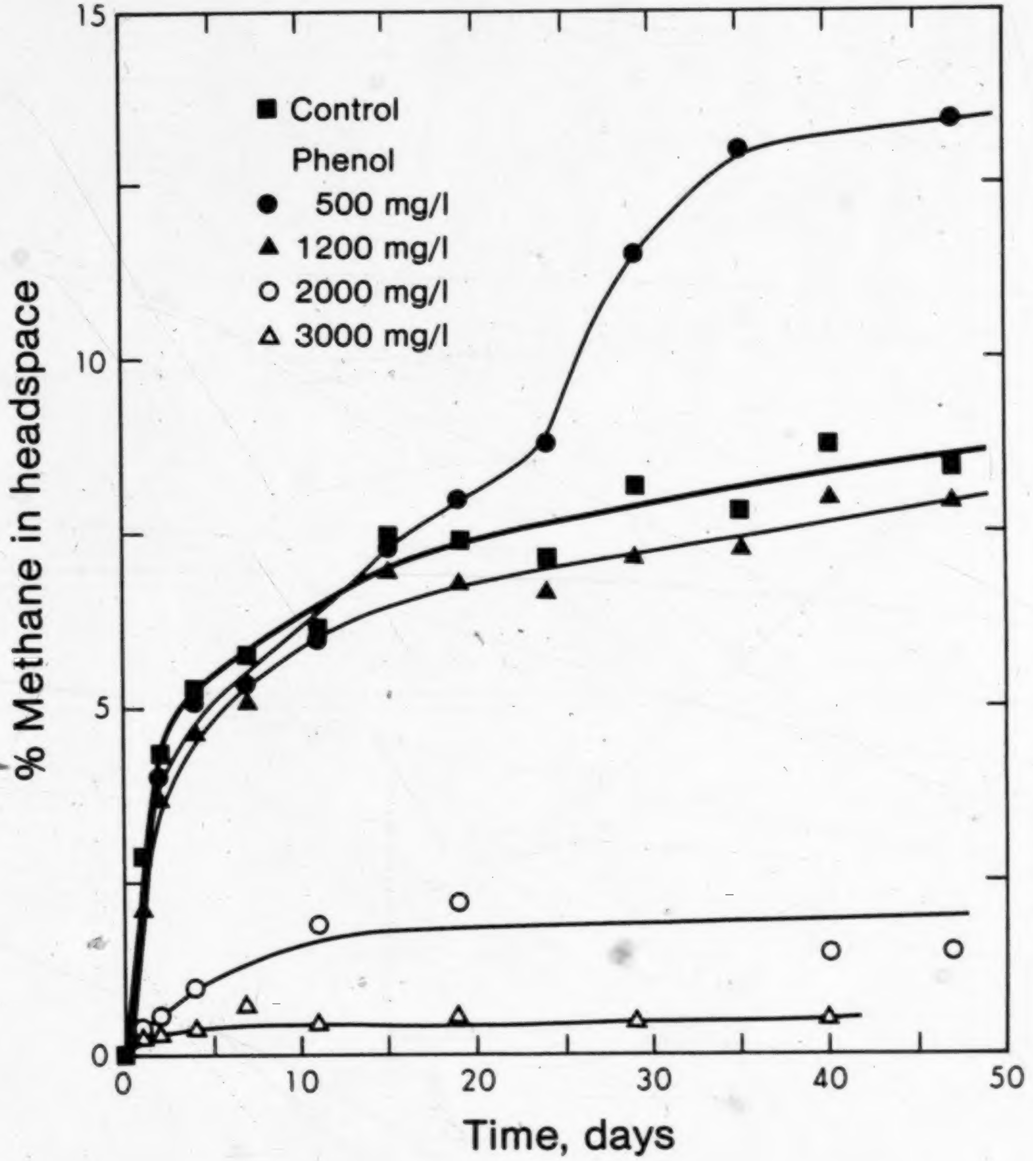


Figure 5.4 Methane production in batch cultures containing various concentrations of phenol - with VOA supplementation.

varying p-cresol concentrations (Figure 5.5 and 5.6). Without VOA-supplementation, (Figure 5.5), the cultures with 200, and 400 mg/L p-cresol produced more methane than the controls after about 20 and 30 days acclimation respectively. A concentration of 1 000 mg/L showed some inhibition while 2 000 mg/L almost completely inhibited methane production. The VOA-supplemented cultures (Figure 5.6) again showed rapid methane production during the first four days of incubation. Concentrations of p-cresol up to 600 mg/L did not inhibit the methane fermentation but 1 000 mg/L produced some inhibition while 2 000 mg/L nearly eliminated the fermentation. After 24 days incubation the cultures containing 200 mg/L p-cresol achieved methane concentrations significantly ($P<0.05$) different from control cultures, and after 27 days the 400 mg/L cultures did the same.

These batch cultures indicate that methane bacteria and many of the non-phenolic-degrading acid-formers (Figure 5.2) are not affected by p-cresol concentrations up to 600 mg/L, however, the p-cresol-degrading acid-formers are inhibited at 600 mg/L. As shown by the VOA-supplementation experiments, the methane bacteria are slightly inhibited at 1 000 mg/L p-cresol and greatly inhibited at 2 000 mg/L.

In their studies on the biokinetics of anaerobic phenol degradation, Neufeld _et_ _al_. (1980) found no methane production in their draw and feed cultures operated at substrate concentrations between 200 and 1 200 mg/L. They suggested that the relatively fragile methane producers

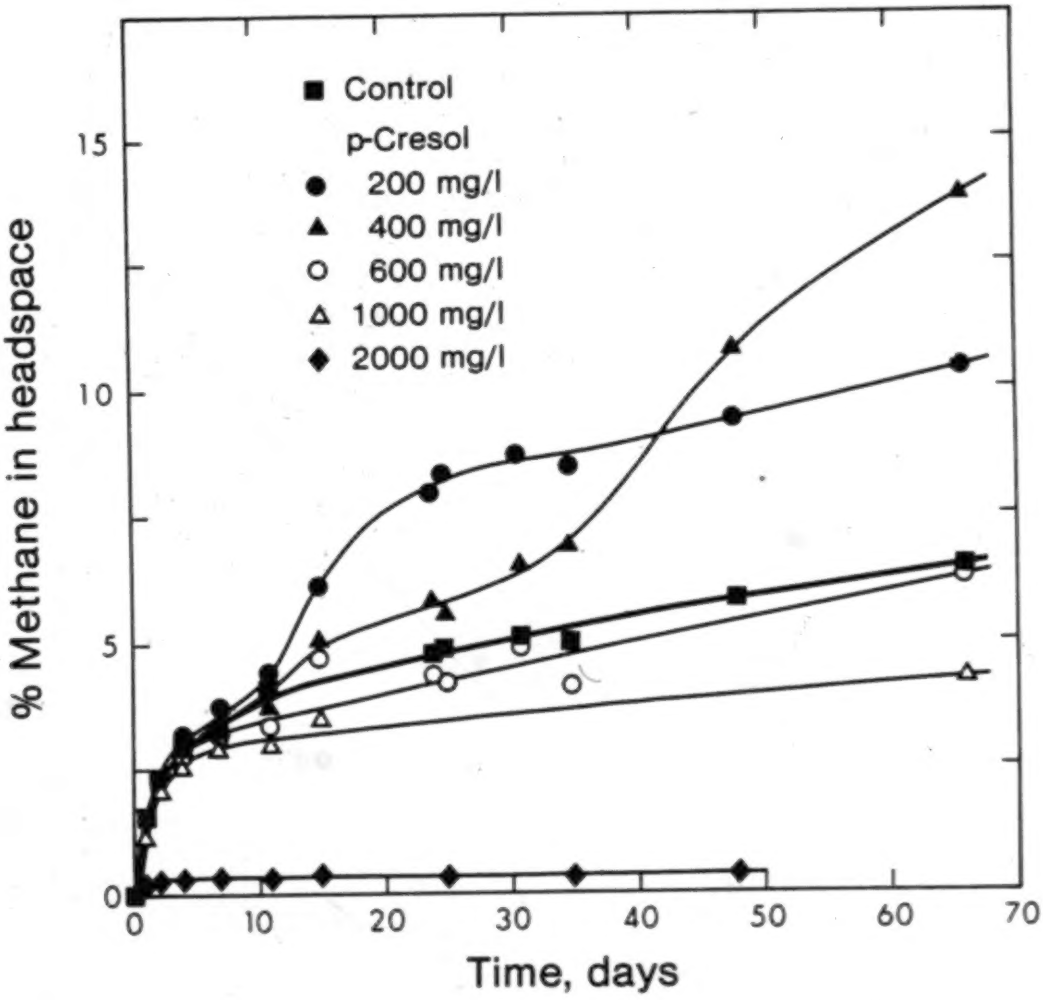


Figure 5.5 Methane production in batch cultures containing various concentrations of p-cresol - without VOA supplementation.

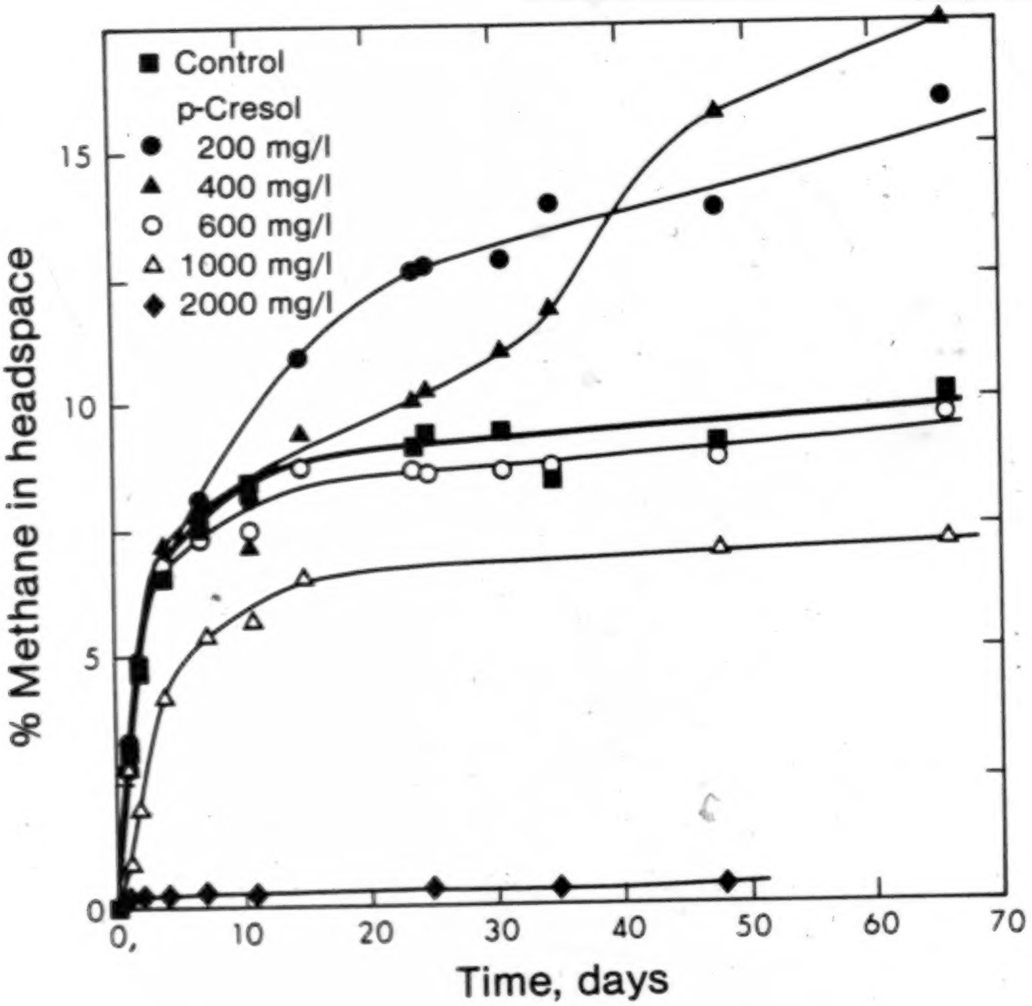


Figure 5.6 Methane production in batch cultures containing various concentrations of p-cresol - with VOA supplementation.

could not grow in the presence of the high phenol levels tested. However, these data (Figure 5.4) clearly show that the methanogens are not inhibited by phenol at concentrations up to 1 200 mg/L in batch cultures. Therefore, the total absence of methane production reported by Neufeld _et al_. (1980) would not be attributable to methanogen inhibition according to our findings.

The hypothesis that the non-phenolic-degrading acid-formers (Figure 5.2) were not inhibited at intermediate concentrations of the fermentable phenolics (i.e. 800 to 1 200 mg/L phenol and 600 mg/L p-cresol) was based on the assumption that the batch cultures contained fermentable complex organics which were introduced with the sludge inoculum. There was a remote chance that the sludge contained only VOAs and that the non-phenolic-degrading acid-formers were not active at all because of a lack of substrate - thereby invalidating the conclusion of their tolerance to these concentrations of the phenolics.

To clarify this point, another two series of supplemented batch cultures were established. One series contained varying concentrations of phenol while the other contained varying concentrations of p-cresol. All of the cultures containing the phenolic substrate were supplemented with NBG (nutrient broth as a source of protein, and glucose as a fermentable carbohydrate). One set of control cultures contained NBG but no phenolic compound while another set contained neither NBG nor the phenolic.

The methane production by the phenol-containing cultures is shown in Figure 5.7 and that by the p-cresol-containing cultures is shown in Figure 5.8. Throughout the incubation period, the controls containing NBG produced higher concentrations of methane than did the unsupplemented controls. By day 47, the headspace gas in the former set contained 6.4% methane while that in the latter set contained only 3.9%. These results show that within the supplemented batch cultures there was an active population of non-phenolic-degrading acid-formers. Also, cultures with 500 mg/L phenol (Figure 5.7) and those with 200 and 400 mg/L p-cresol (Figure 5.8) showed enhanced methane production indicating that the sludge inoculum contained an active population of phenolic-degrading acid-formers.

When phenol was present at 800 mg/L there was no inhibition of methane production in the NBG supplemented cultures (Figure 5.7) and in fact, enhanced methane production was observed after 33 days incubation. This was the highest concentration of phenol observed to be fermentable during the entire project. When phenol was present at 1 000 mg/L the methane concentrations observed on eight of the 15 sampling days were slightly lower (<8%) than those in the NBG-supplemented controls. On 12 of the 15 sampling days, the cultures containing 1 200 mg/L phenol had significantly less methane than the control (Figure 5.7). However this difference was never less than 15% of the NBG-containing control value and the observed concentrations

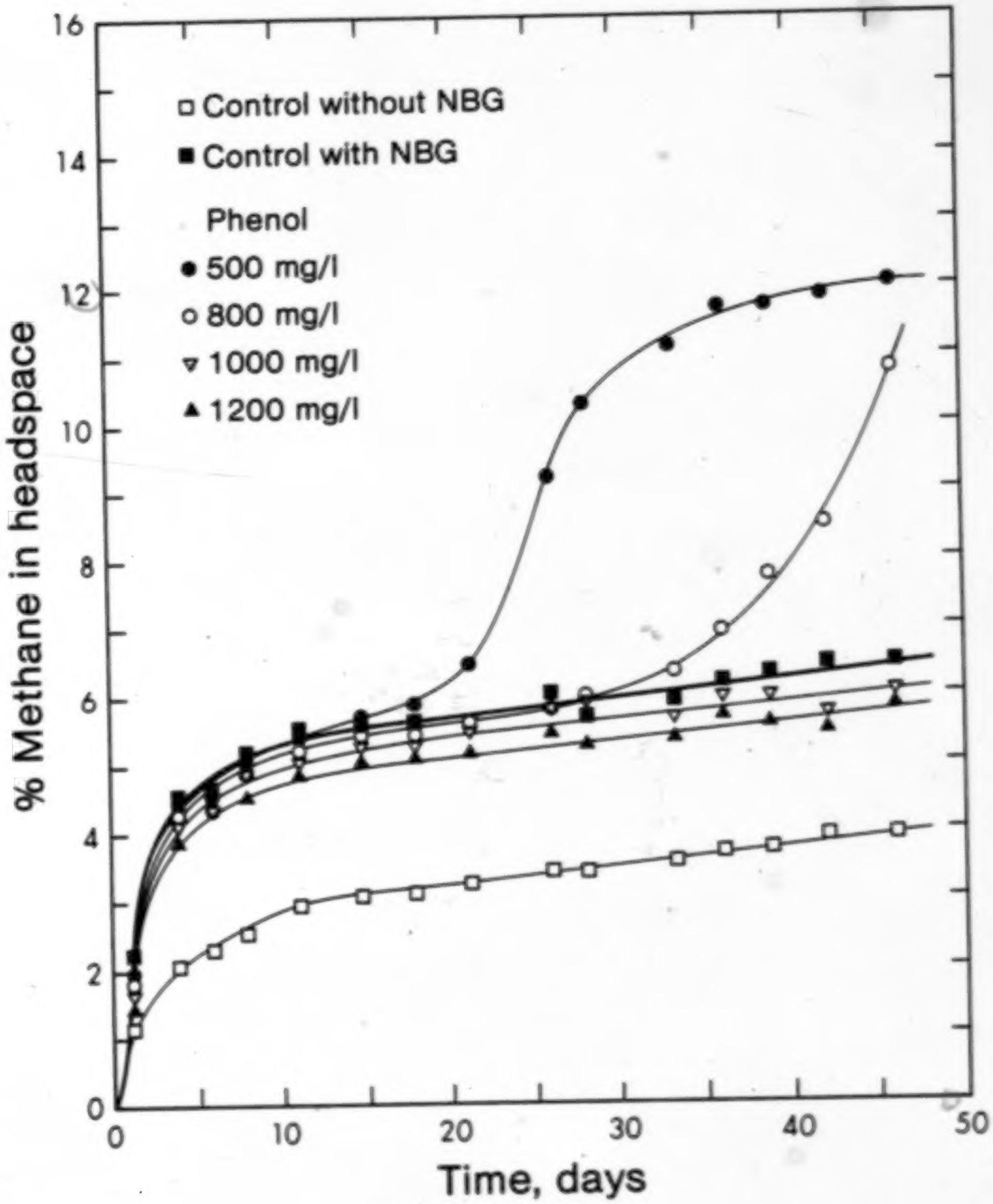


Figure 5.7 Methane production in batch cultures containing various concentrations of phenol - supplemented with nutrient broth and glucose (NBG).

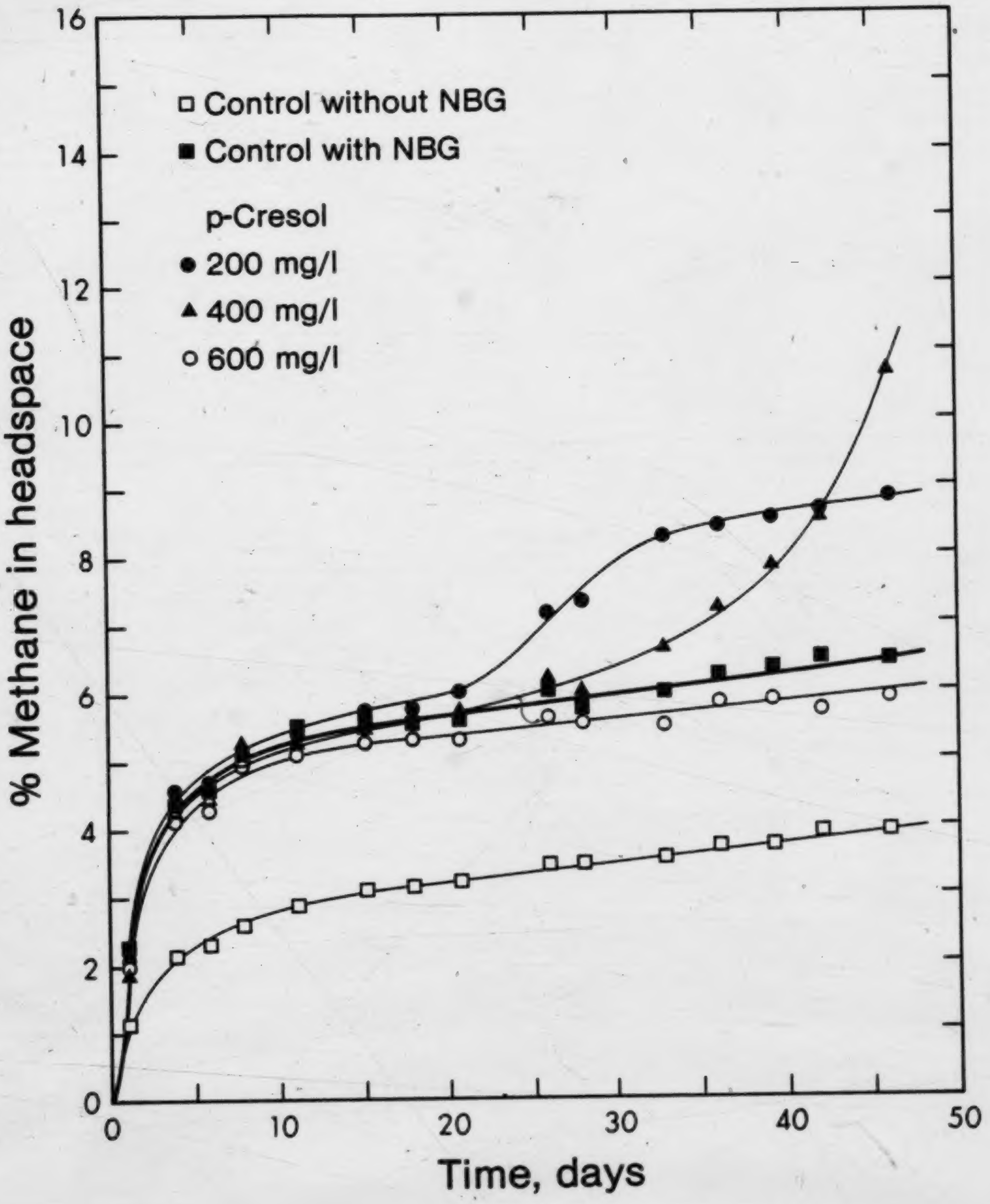


Figure 5.8 Methane production in batch cultures containing various concentrations of p-cresol - supplemented with nutrient broth and glucose (NBG).

of methane in the 1 200 mg/L cultures were always much greater than those in non-NBG-supplemented controls. These results clearly show that the non-phenolic-degrading acid-formers are less sensitive to the elevated phenol concentrations than are the phenolic-degrading acid-formers (Figure 5.2).

Similar results were observed with p-cresol at 600 mg/L (Figure 5.8). In this case, on 11 of the 15 sampling days, the concentrations of methane in these cultures were significantly lower than those in the NBG-supplemented controls. However, this difference was never greater than 10% of the NBG control value. As was observed with phenol, when the concentration of p-cresol was high enough to inhibit the phenolic-degrading acid-formers (600 mg/L) there was little inhibition of the non-phenolic-degrading acid-formers, as indicated by the higher concentrations of methane in these NBG-supplemented cultures than in the non-supplemented cultures.

5.2.3 Substrate Loss From Methane Producing Cultures

To this point in the discussion, it has been assumed that enhanced methane concentrations in batch cultures containing phenol at ≤500 mg/L or p-cresol at ≤400 mg/L has been the result of biodegradation of these compounds. Figures 5.9 and 5.10 verify this assumption by showing the loss of substrate and subsequent methane production. A batch culture was established with an initial phenol concentration

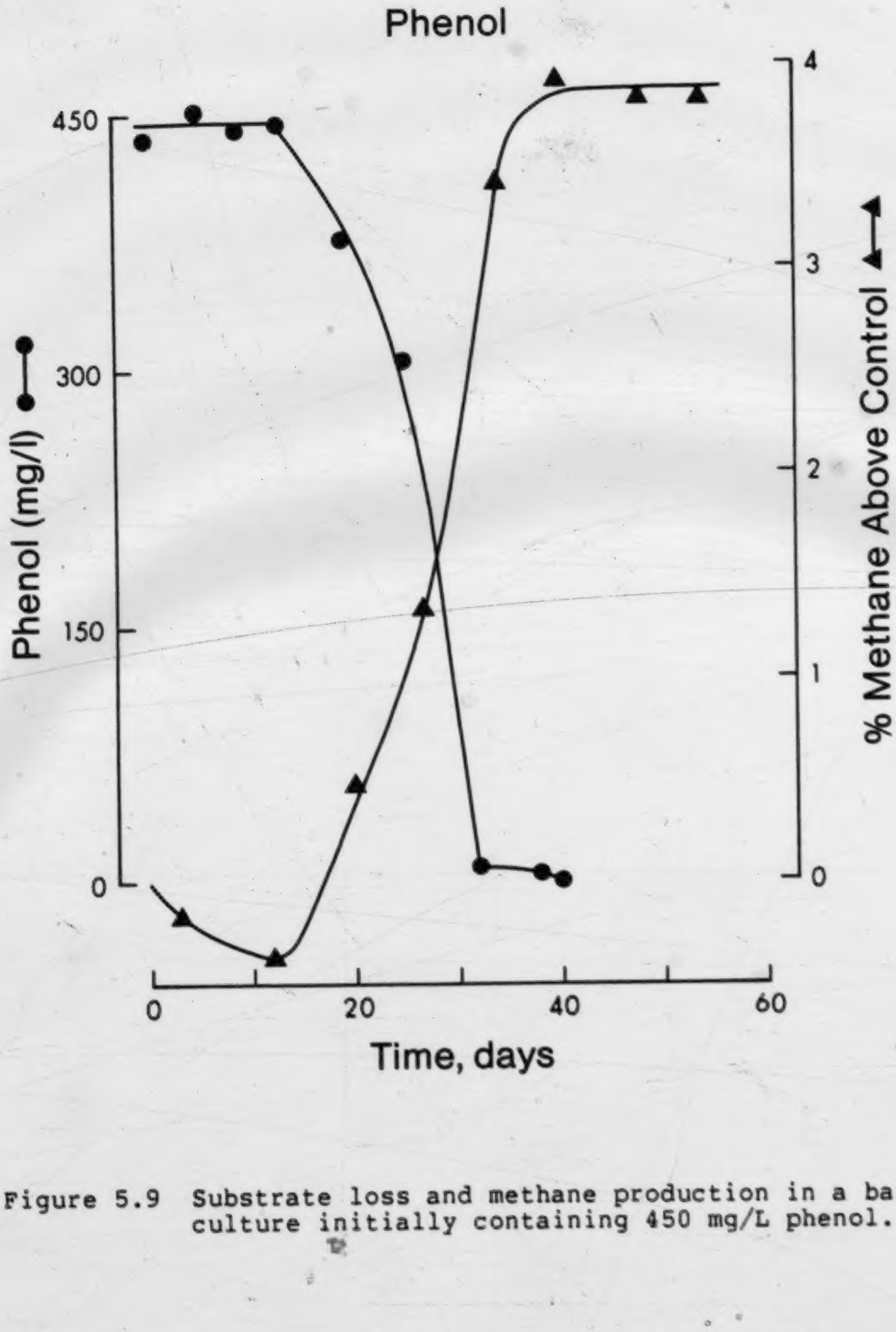


Figure 5.9 Substrate loss and methane production in a batch culture initially containing 450 mg/L phenol.

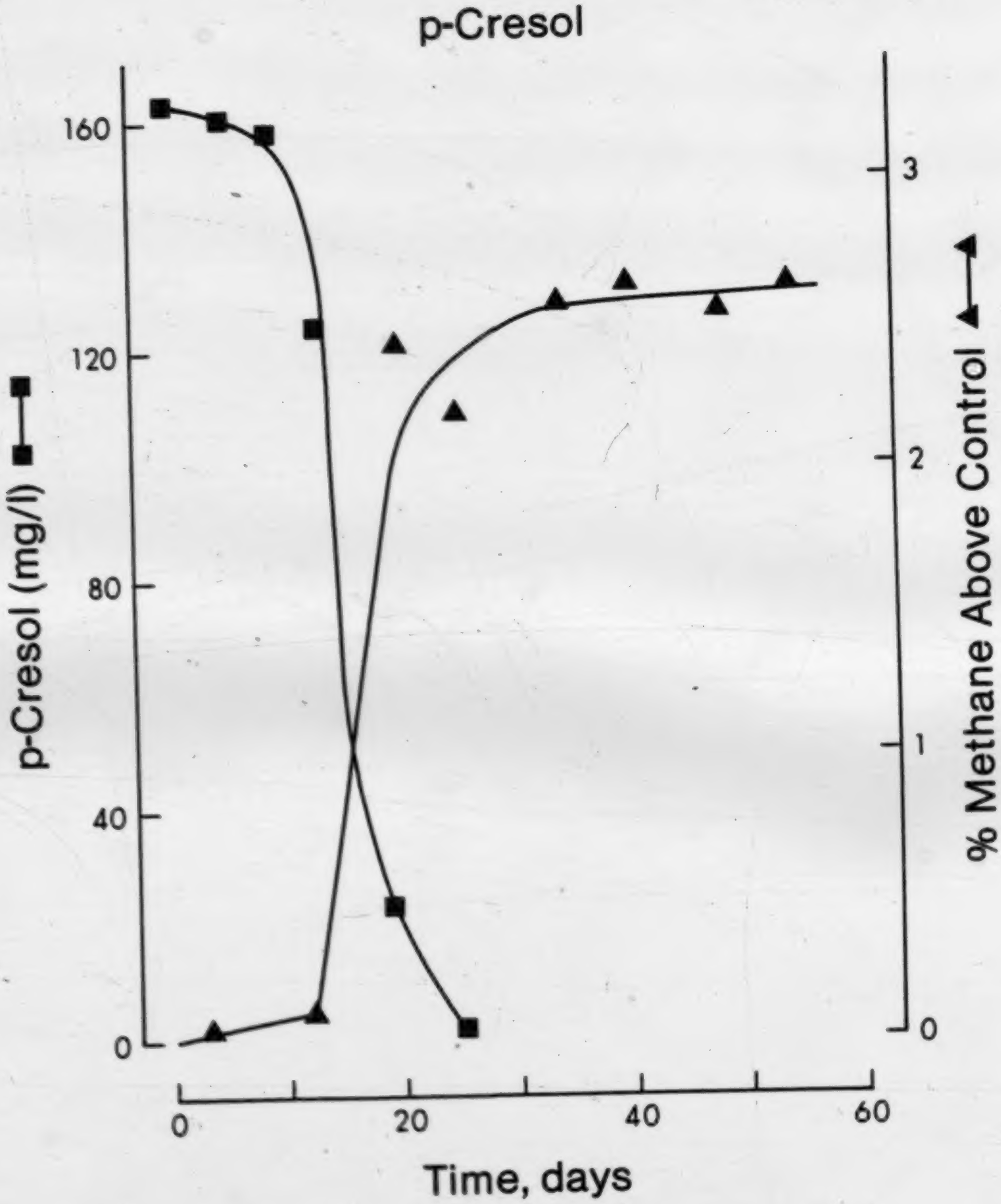


Figure 5.10 Substrate loss and methane production in a batch culture initially containing 160 mg/L p-cresol.

of 450 mg/L. After an acclimation time of 16 to 20 days, the concentration of phenol decreased quickly until day 32 when there was virtually none left in the culture (Figure 5.9). Methane analyses on this culture and a control culture (containing no phenol) showed that by day 20, when the concentration of phenol began to decrease, methane production in excess of the control was observed. Methane production continued until shortly after phenol had been depleted from the culture.

Figure 5.10 shows similar results for a batch culture which initially contained 160 mg/L p-cresol. Between days 10 and 16 there was a significant loss of p-cresol and as this substrate continued to be degraded, there was a corresponding increase in the concentration of methane in the headspace gas. The methane level reached a plateau after the p-cresol was no longer detectable.

Thus, the measurement of methane production in excess of that in a non-phenolic containing control culture can be used as a good indication of the degradation of these phenolic substrates which have been shown to ferment to methane. Since the methane analysis was much quicker than the substrate analysis, cultures were routinely monitored for methane production rather than substrate loss.

5.2.4 Fermentable Substrate Concentration and Acclimation Time

The data in Figures 5.5, 5.6 and 5.8 show that the cultures containing 200 and 400 mg/L p-cresol both fermented that substrate, but the culture with the higher substrate concentration required a longer acclimation time. Consequently, a series of cultures with varying concentrations of either phenol or p-cresol were inoculated to test whether the phenolic concentration did affect the acclimation time. The findings of two such experiments are summarized in Table 5.4 which verify that the higher the concentration, the longer the acclimation time. There also appears to be a threshold concentration, below which the acclimation period is fairly short, and above that concentration, acclimation takes longer. This was especially evident in the case of phenol where the threshold appears to be between 300 and 400 mg/L. At 300 mg/L or less, acclimation required 14 to 15 days with the two sludge samples used for inoculation. In trial 1, increasing the concentrations to 400 and 500 mg/L increased the acclimation times to 18 and 26 days, respectively, while in trial 2, times of 16 and 19 days were required for acclimation to these two phenol concentrations.

In trial 1 (Table 5.4), the responses of the batch cultures to increasing concentrations of p-cresol were not as orderly as were those of phenol-containing cultures nor the p-cresol-containing cultures in trial 2. Some

Table 5.4 Effects of fermentable phenolic substrate concentration on acclimation time.

Substrate	Concentration (mg/L)	Time at which methane concentration was greater than in control (days)	
		Trial 1	Trial 2
phenol	100	15	14
	200	15	14
	300	15	14
	400	18	16
	500	26	19
p-cresol	100	15	14
	200	>39(2)	18
	300	39	21
	400	39	31

(1) When the average methane concentration in the three culture bottles was significantly ($P>0.05$) higher than that of the three control cultures.

(2) Two of the three cultures were acclimated within 22 days, but the third required >39 days to adapt.

variability in the responses of the microbial populations from different sludge samples would be expected and was observed. For example, one of the three cultures given 200 mg/L p-cresol required over 39 days to adapt to the fermentation of this substrate, while the other cultures required only 22 days. Those cultures were all inoculated with aliquots from the same sample of municipal digester sludge. Variable responses were also evident when cultures were established using sludge sampled on different days. This is most evident when the acclimation times from trial 2 were compared to those from the first trial. The sludge used as the second inoculum fermented p-cresol much more readily than did the first sludge and the observed acclimation times increased as the substrate concentration was increased.

Thoughout this project, there were more difficulties establishing active cultures on p-cresol than on phenol. Table 5.4 shows that for concentrations greater than 100 mg/L, the acclimation time for p-cresol was always longer than that for phenol. During the initial screening experiments (Table 5.2) p-cresol was not fermented when it was present at 400 mg/L. However, other data presented (e.g. Figures 5.5, 5.6 and 5.8 and Table 5.4) clearly show that it is fermentable at this concentration when different sludge samples were used for inoculation. These data suggest that the phenolic-degrading organisms are numerically only a minor portion of the microbial population of the sludge since if they were in great abundance, less variation between samples should be observed. The issue of phenol-degrader population is addressed in chapter 6.

This series of laboratory scale batch cultures provides insight into the effects and possible designs of digesters receiving phenolic wastewaters. If an operating digester were to receive a shock load of phenol or alkyl phenolics it would likely not upset the overall process unless the concentrations, after dilution in the digester, were very high. The majority of the phenolic compounds tested here, would pass through the digester unaltered. Based on the results from this series of batch cultures, only phenol and p-cresol would be fermented to methane provided they were at concentrations which did not inhibit the phenolic-degrading acid-formers (Figure 5.2) and that their retention time in the digester was adequate to allow the microorganisms to adapt to them.

The long acclimation periods required for the fermentable phenolics indicate that a process which provides a long mean cell residence time, such as a fixed film system, would be best suited to treat wastewaters containing these compounds. During the start up of such a system, the phenolics should be introduced gradually to minimize the overall acclimation time.

5.2.5 Ultimate Gas Production From Phenol and p-Cresol

Figures 5.9 and 5.10 show that methanogenic cultures can completely remove phenol and p-cresol from culture fluid with the subsequent production of methane. The extent to which substrate-carbon is converted to methane and carbon dioxide in batch culture can be considered as the ultimate amount of gas produced. Knowing this value, the overall performance of semicontinuous or continuous cultures can be evaluated by monitoring their gas production rates.

Buswell and Muller (1952) have proposed the following expression to predict the amounts of methane and carbon dioxide from the fermentation of a compound consisting of carbon, hydrogen and oxygen.

$$C_nH_aO_b + \left[n - \frac{a}{4} - \frac{b}{2}\right]H_2O \longrightarrow \left[\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right]CO_2 + \left[\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right]CH_4 \qquad (5.2)$$

This equation is widely used to judge the extent of substrate carbon conversion in anaerobic cultures.

Batch cultures were established containing accurately known amounts of either phenol or p-cresol and the volumes of methane and total gas were determined. These measurements were corrected to account for the presence of water vapour and the volumes were corrected to standard conditions (STP). The experimentally measured volumes were then compared to Buswell's stoichiometric predictions.

Figure 5.11 shows the methane production observed from two separate trials. In each experiment, three pairs of phenol-containing and control cultures were monitored. Table 5.5 compares the results of these with the stoichiometric

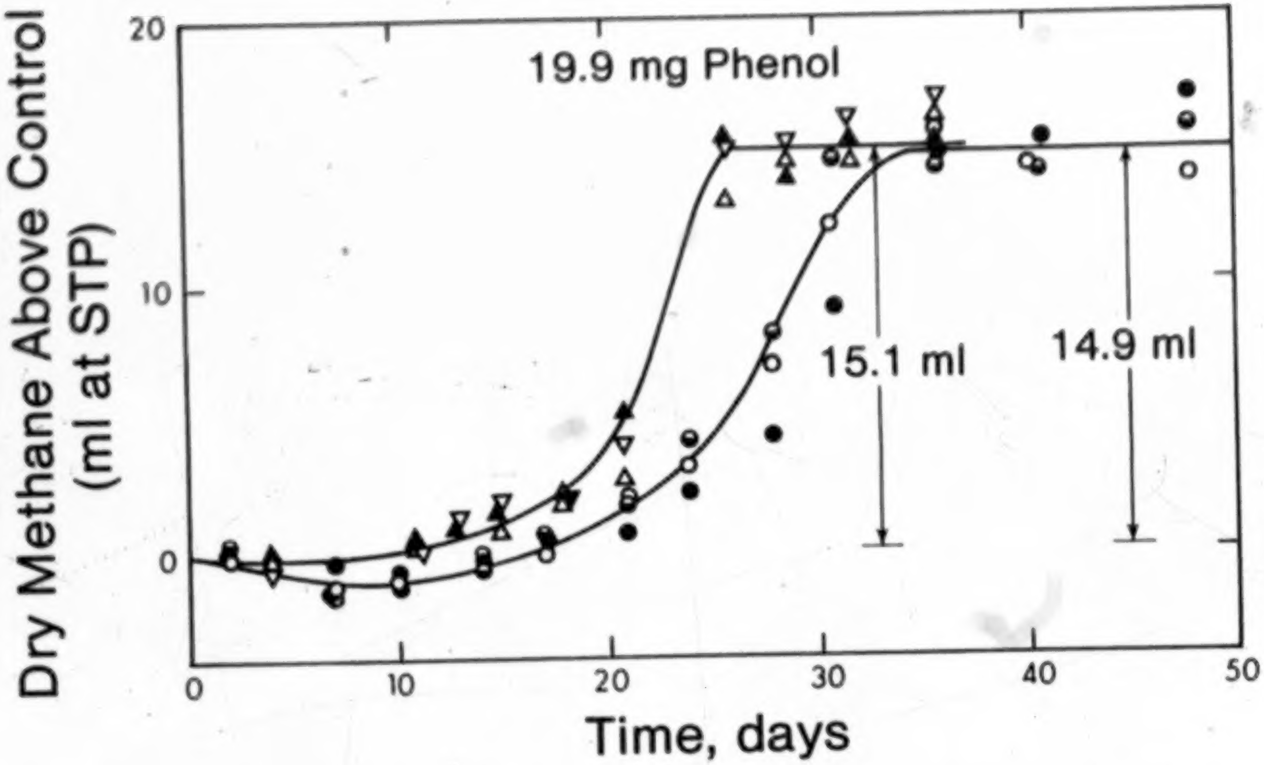


Figure 5.11 Ultimate volume of methane produced from phenol in batch cultures.

Table 5.5 Ultimate methane and total gas production from phenol and p-cresol.

Substrate	Methane			Total Gas		
	Predicted (mL)	Found (mL)	Percent of Predicted	Predicted (mL)	Found (mL)	Percent of Predicted
Phenol[1]						
Trial 1	16.7	15.1	90.4	28.6	22.9	80.0
Trial 2	16.7	14.9	89.3	28.6	21.9	76.6
Healy and Young (1978)	--	--	88.7	--	--	79.0
p-Cresol[2]						
Trial 1	13.1	11.3	86.3	21.6	17.3	79.8
Boyd *et al.* (1983)	--	--	>90	--	--	--

[1] Cultures contained 19.9 mg phenol

[2] Cultures contained 14.9 mg p-cresol

predictions and with similar values reported in the literature. Buswell's equation for phenol is:

$$C_6H_6O + 4H_2O \rightarrow 2.5CO_2 + 3.5CH_4 \qquad (5.3)$$

Thus 1 mmole phenol should give (22.4 mL/mmole x 3.5 mmole) or 78.4 mL CH_4. Therefore 19.9 mg phenol (0.213 mmole) should give 16.7 mL CH_4. The volumes of methane found in the two experiments were 15.1 and 14.9 mL which correspond to 90.4% and 89.3%, respectively, of the predicted volumes. The expected total gas production was 28.6 mL and the observed amounts were 22.9 and 21.9 mL. These are 80.0% and 76.6%, respectively, of the predicted amounts. Healy and Young (1978) reported the actual amounts of methane and total gas observed from their cultures to be 88.7% and 79.0%, respectively, of the stoichiometric amounts. The values presented in Table 5.5 are in close agreement with these published values, thereby illustrating the reliability of the analytical methods used in the present study.

Methane production in batch cultures containing 14.9 mg p-cresol is shown in Figure 5.12. Because of experimental difficulties, only data from two of the three pairs of cultures were reliable. The predicted gas volumes in Table 5.5 are based on the equation:

$$C_7H_8O + 4.5H_2O \rightarrow 2.75CO_2 + 4.25CH_4 \qquad (5.4)$$

The amount of methane found was 86.3% of the predicted amount while the total gas volume was 79.8% of the expected volume. The only comparable literature value available was

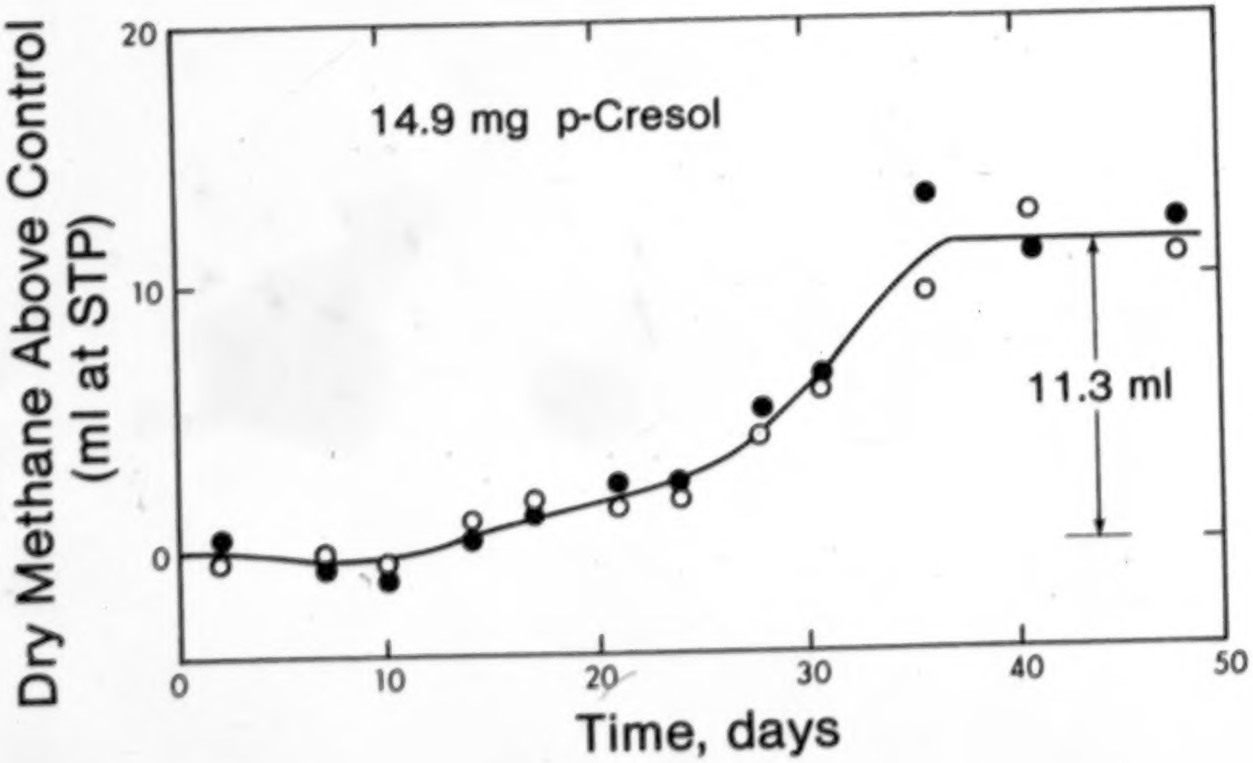


Figure 5.12 Ultimate quantity of methane produced from p-cresol in batch cultures.

given by Boyd et al. (1983) who reported >90% of the predicted amount of methane was formed.

The observed total gas volumes in Table 5.5 are all about 20% less than those predicted by Buswell's equation. This is not entirely unexpected since the stoichiometric equation does not account for any of the metabolized carbon being incorporated into microbial mass. In fact, McCarty (1964) and Kirsch and Sykes (1971) report that 10% to 20% of substrate carbon is converted to cell mass under anaerobic conditions.

Methane and carbon dioxide account for essentially all of the total volume of gas produced by methanogenic consortia. Thus the predicted and observed volumes of carbon dioxide can be estimated from the data in Table 5.5. The predicted volumes from phenol and p-cresol fermentation are 11.9 and 8.5 mL, respectively. The mean carbon dioxide volumes found were 7.4 and 6.0 mL from phenol and p-cresol, respectively. Thus, for phenol, the observed volume was 62.2% of the predicted value and for p-cresol, it was 70.6%. These are much lower that the recoveries of methane which were >86% of the expected volumes. Two factors likely account for this discrepancy. First, carbon dioxide is more soluble in water than methane. It is possible that a small amount remains in the aqueous phase while the volume of the headspace gas is being measured. However, since the culture was initially in equilibrium with a 30% carbon dioxide atmosphere in the headspace, it is unlikely that solubility was the cause of the low recoveries of carbon dioxide. Second, and more importantly, under anaerobic conditions, carbon which exists as methane is unavailable for cell growth. That is, once formed, methane is biologically inert under anaerobic conditions. In contrast, some anaerobes, such as sulfate reducing bacteria, some methanogens and some *Clostridium* spp. assimilate carbon from carbon dioxide (Gottschalk, 1981). Thus the expected proportion of carbon dioxide recovered from these batch cultures would be lower than the proportion of methane recovered.

5.2.6 The Ability of Alternate Sources of Inocula to Degrade Phenolics

Of the ten phenolics screened for fermentability using domestic anaerobic sewage sludge as an inoculum (Table 5.2), only phenol and p-cresol were fermented to methane. These are both intermediates in the anaerobic degradation of the amino acid tyrosine (Balba and Evans, 1980b; Barker, 1981) which would be found in domestic sludges. Thus tyrosine would provide an enrichment for phenol and p-cresol degrading bacteria in this environment. The other alkyl phenolics tested would not likely be found in significant concentrations in domestic sludges which were used in this study and by others (Chmielowski et al., 1965; Suidan et al., 1981). However, other anaerobic sludges or muds which have been exposed to these alkyl phenolics may contain populations which are able to ferment these compounds. Three such sludges were tested in this study.

One sludge originated from the 15 m depth of a tar sands tailings pond. In the water from this pond, Hargesheimer (1981) found phenol, the three isomers of cresol and four different dimethyl or ethylphenols at concentrations below 0.1 mg/L. She also observed that phenol was rapidly metabolized during sample storage. Although this degradation was likely due to aerobic metabolism in her samples, it is possible that anaerobes may also be active in the pond. Since there was no prior information on whether methane bacteria were present in this sludge, the test cultures were co-inoculated with domestic anaerobic sludge. Thus, if the tailings pond sludge contained anaerobic non-methanogens which were capable of degrading the test phenolics to VOAs, the presence of the domestic sludge would assure methane production.

The results of trial 1 showed that when readily fermentable substrates were added to these batch cultures (i.e. glucose, VOAs, phenol, and p-cresol), methane production in excess of that in the control cultures was observed. However, none of the other eight phenolics tested (Table 5.1) were found to be fermentable over a 95-day incubation period.

For the second trial with this sludge, the size of the inoculum was tripled to increase the likelihood of detecting anaerobic phenolic-degraders. However, as with the first trial, only phenol and p-cresol were found to be fermentable during the long (105 day) incubation period.

The first Dearborn sludge originated from a laboratory scale anaerobic filter treating coking plant wastewaters. This sludge remained viable during transport. Within the first day of incubation, methane concentrations in excess of the control were observed from glucose, the VOAs and the phenolic wastewater to which it was acclimated. However, over the 71-day incubation period, among the 13 phenolics tested, only phenol and p-cresol were fermented to methane.

When received in our laboratory, the second Dearborn sludge was actively producing gas in the sample bottle. GC analysis of the aqueous portion of the sludge showed 380 mg/L phenol, 16 mg/L o-cresol, 99 mg/L m/p-cresol and lesser amounts of three peaks that had the same retention times as isomers of dimethylphenol.

In the experimental batch cultures, this sludge was very slow to produce methane. The most active cultures were the controls which received no added substrates. Even the glucose-containing positive control cultures produced only slightly more methane than the control in the first eight days. Those cultures which received fermentable substrates at 200 mg/L (i.e. phenol, p-cresol, catechol, and resorcinol), produced significantly less methane over the first 36 to 47 day incubation and never produced more methane than the controls during the 99 day test period. The presence of the other test phenolics also inhibited methane production to varying degrees. The greatest inhibition was found with 1-naphthol and 2-naphthol, each at 100 mg/L, and p-ethylphenol at 200 mg/L. None of the 15 phenolics tested gave enhanced methane concentrations. The cultures given the H-coal wastewater (which had been fed to the reactor in Burlington) at 4% (V/V) produced less than 30% of the amount of methane found in the control culture after 99 days.

The active gas production observed in the sample bottle upon its arrival, may have been due to the fermentation of residual starch since this sludge was being fed a mixture of wastewaters from an H-coal conversion operation and a starch processing plant. However, if this was the case, a quicker fermentation of glucose in the test culture would have been expected. Since the anaerobic filter at the Wastewater Technology Centre was producing methane, but the inoculum would not ferment any of the added phenolics, it is likely that the sludge contained a phenolic-tolerant population in which the non-phenolic-degrading acid-formers and methane bacteria (Figure 5.2) were active but the phenolic-degrading acid-formers were not. This would mimic the situation in the NBG fed cultures (Figure 5.7 and 5.8) which received phenol at 1 000 mg/L or p-cresol at 600 mg/L.

The reason for using the alternate sources of inocula in these batch cultures was to determine whether any other alkyl phenolics could be shown to be degradable under anaerobic conditions. The data from the four attempts described above indicate that, other than phenol and p-cresol, none of the tested phenolics were fermented to methane. These observations suggest that the anaerobic treatment process could not effectively purify wastewaters containing large numbers of phenolic compounds in nearly equal proportions. It may however, be applicable to wastewaters which contain primarily phenol and/or p-cresol. This composition is typical of many industrial wastewaters (Table 2.2).

The failure of these three different sludge samples to yield microbial populations which could degrade a wide range of phenolics suggests that the domestic anaerobic sewage sludge was an acceptable inoculum to use routinely in this study.

The methods used to determine the biodegradability of various alkyl phenolics have three limitations. First, the experiments were set up to evaluate the fermentability of the phenolic to methane. It is possible that some of the compounds were partially metabolized without the formation of methane. Since the substrate concentrations were not monitored, such changes could not be detected. Second, in order to detect fermentation of the phenolics, the amount of substrate present in the cultures had to be large enough that the resulting methane would be detectable above the background methane concentrations produced in the controls which were devoid of phenolics. Substrate concentrations of ≥100 mg/L were typically chosen as the lowest test level to ensure adequate methane production. It is possible that the lowest concentration tested might be inhibitory to the specific organisms which are able to degrade the substrate and that lower concentrations would be degradable. Third, the phenolic-containing cultures were typically monitored for only 40 to 60 days. Longer acclimation times may be required for the degradation of some of the alkyl phenolics, This requirement was found for m-cresol and is discussed in chapter 7.

Considering these three aspects in light of assessing the treatability of the alkyl phenolics by anaerobic methods, the above limitations do not invalidate the results which were presented. For example, if the carbon from an organic compound remains in intermediates rather than being converted to methane and carbon dioxide, the overall removal of BOD is relatively small and the treatment efficiency is low. Also, since the concentrations of these alkyl phenolics are often quite high in wastewaters from energy-related industries (Table 2.2), the initial test levels of 100 to 200 mg/L were not unreasonable. Finally, if extremely long acclimation times are required for a given compound, the start-up and stable operation of a large scale anaerobic treatment process for the removal of that compound may not be practical.

6. FERMENTATION OF PHENOL IN BATCH CULTURES

Process design of any wastewater treatment system ultimately requires knowledge of the pollutant removal kinetics. The work reported in this chapter studied the rate of and factors affecting the degradation of phenol by methanogenic consortia. These investigations evaluated whether the microbial process kinetics could be accurately represented by existing substrate removal kinetic models. The results indicate that the processes involved are complex and are not readily described by simplified models. However, the results do provide some useful observations on the overall behaviour of the microbial consortia required to degrade phenolics with the generation of methane.

The initial experiment monitored the rate of phenol loss from a series of batch cultures containing various initial substrate concentrations. The results were evaluated to determine whether these batch cultures followed the Monod kinetic model (Monod, 1949). The shape of the substrate loss curves from these cultures suggested that at the start of phenol removal some factor was limiting the degradation rate. Hydrogen was thought to be limiting since it is required during the initial metabolism of the aromatic compound (Evans, 1977). Two batch culture experiments were done to determine whether the acclimation time could be shortened and/or the initial phenolic degradation rate could be increased by increasing the availability of molecular hydrogen in the culture. In the first experiment, propionate, which is converted to acetate, CO_2 and H_2, was added to the cultures while in the second experiment H_2 was added directly.

A most probable number (MPN) method was used to enumerate phenol-degrading units (PDU) in anaerobic sludge samples. The results from preliminary attempts are presented along with a discussion showing that the method appears to greatly underestimate the actual number of phenol-degrading organisms within the consortia.

6.1 Procedures

6.1.1 Kinetics of Phenol Degradation in Batch Cultures

Sixteen batch cultures containing varying concentrations of phenol were established using 5 mL aliquots of domestic anaerobic sewage sludge as inoculum. These were added to serum bottles which contained 4 mL medium, 1 mL of phenol solution prepared at 10 times the desired final concentration and 0.1 mL sodium sulfide solution. The final phenol concentrations in the inoculated cultures were approximately 50, 65, 80, 90, 100, 120, 150, 200, 300, 400, 500, 550, 600, 650, and 700 mg/L. Duplicate cultures were prepared with 65 mg/L phenol and five control cultures, containing no phenol, were incubated along with the test cultures.

Phenol concentrations were determined by GC analysis using direct aqueous injections. These were done daily for cultures which initially contained ≤100 mg/L phenol and twice daily after phenol degradation had begun. Those cultures which initially contained >100 mg/L were analyzed less frequently prior to the start of phenol degradation and then once or twice daily after its loss was detected. The original substrate concentration in each culture was taken as the average of the GC measurements prior to the start of degradation. Methane analyses were done routinely to ensure that the methanogenic consortium was active in the batch cultures.

At the time of inoculation, six aliquots of 5 mL each were used to determine the volatile nonfiltrable residue (volatile suspended solids) content of the sludge using method 209 G (APHA, 1980). After the batch cultures had degraded the phenol and methane production had stopped, the volatile nonfiltrable residue in each culture was determined using 8 mL aliquots from each bottle. The residue analysis was also done on control cultures.

6.1.2 Propionate-supplemented Batch Cultures

Twenty-four serum bottles containing 4 mL medium and 1 mL phenol solution (3 000 mg/L) were prereduced and inoculated with 5 mL domestic anaerobic sewage sludge. Similarly, three control cultures containing water in place of the phenol solution were inoculated and these did not receive sodium propionate supplementation.

Two prereduced solutions of sodium propionate were prepared in bicarbonate buffer as follows. A 1 mL aliquot of resazurin solution was added to 99 mL water and this was boiled to remove dissolved O_2. Then 0.57 g $NaHCO_3$ was added and the solution was allowed to cool while being bubbled with O_2-free 30% CO_2 in N_2. Suitable volumes of this solution were added to gassed serum bottles containing preweighed amounts of sodium propionate which gave solutions of 19 800 mg/L and 38 400 mg/L of this salt. These were reduced with 0.1 mL sodium sulfide solution per 10 mL propionate solution prior to their addition to the cultures.

Immediately after the cultures were inoculated, three of them received 0.1 mL of the 19 800 mg/L sodium propionate solution and three others received 0.1 mL of the 38 400 mg/L solution. One set of three phenol-containing cultures was designated as a second control series which did not receive sodium propionate. Another group of three bottles was supplemented daily with 10 μL aliquots of 19 200 mg/L sodium propionate during the entire duration of the experiment.

All of the cultures were incubated at 37°C and the phenol concentrations were measured daily while methane analyses were done two or three times each week.

After nine days incubation, three more bottles were supplemented with 0.1 mL 19 800 mg/L sodium propionate and three others received 0.1 mL of the 38 400 mg/L solution. On day 15, when the phenol concentrations began to decrease in the phenol-containing control cultures and the six remaining unsupplemented cultures, the latter six cultures were fed sodium propionate in the same manner as those which were supplemented on day 9.

The mean phenol concentrations for each of the seven sets of propionate-supplemented cultures were compared to that of the non-supplemented, phenol-containing control using the statistical method of Dunnett (1955) to determine whether propionate addition altered the rate of phenol degradation.

6.1.3 Hydrogen-supplemented Batch Cultures

This experiment was set up and the cultures were monitored in essentially the same manner as the propionate-supplemented batch cultures. However, these cultures received injections of H_2 at various times rather than propionate solution. At the time of inoculation, one triplicate set received 0.5 mL H_2 while another set received 1.5 mL. On day 9, three cultures were given 1.5 mL H_2 while three others were given 3.0 mL. When phenol degradation was detected (on day 15) one triplicate series received 0.5 mL H_2, another received 1.5 mL, while a third received 3.0 mL. Throughout the duration of the experiment, one triplicate set of cultures was supplemented daily with 0.15 mL H_2. Two sets of control cultures were maintained with the test cultures. One contained phenol while the second did not. Neither control series was supplemented with H_2.

6.1.4 MPN Method

Four sets (trials 1-4) of MPN analyses were done to determine the number of PDU in anaerobic sludge samples. In the first two trials, suitable aliquots of medium containing 200 mg/L phenol were dispensed into gassed serum bottles, autoclaved and prereduced prior to inoculation. In the last two trials, the medium was prepared without phenol and after inoculation, 0.1 mL prereduced phenol solution (20 000 mg/L) was added to each culture bottle.

In trial 1, triplicate 0.5 mL aliquots of domestic anaerobic sludge were used as initial inocula and these were 10-fold serially diluted in 4.5 mL medium to a final dilution of 10^{-4}. In the second trial, triplicate 1 mL aliquots of domestic anaerobic sludge were used as initial inocula and these were 10-fold serially diluted in 9 mL medium to a final dilution of 10^{-3}. Also in this case, three individual 10 mL aliquots of sludge were placed in separate gassed serum bottles. These were sealed and 0.4 mL of prereduced phenol solution was added to each to give a final phenol concentration of approximately 200 mg/L. This MPN series was designated trial "2a". A 45 ml aliquot of this sludge was combined with 5 mL of a 2 000 mg/L phenol solution as outlined in section 5.1.5. This 50 mL culture was incubated at 37°C until GC analyses showed that the phenol had been removed. Then an MPN dilution series was prepared from this culture in the same manner as was done for trial 2a. This set was designated trial "2b" and was intended to indicate the extent of increase in PDU in a batch culture which consumed 10 mg phenol.

Trial 3 was set up identically to trial 2a except the 5-tube method was used to enumerate PDU in domestic anaerobic sludge. The 5-tube method was also used in trial 4 where aliquots of 1 mL of an active phenol-degrading culture were serially diluted to 10^{-3}. This active culture was initially established with 4 mL medium, 1 mL phenol solution (2 000 mg/L) 2 mL domestic anaerobic sludge and 2 mL of an anaerobic sludge from a sludge blanket reactor treating pulping wastewater (provided by Dr. J.F. Ferguson, University of Washington, Seattle, WA). After the phenol had been removed from this batch culture, the MPN dilutions were made.

All of the MPN cultures originally contained near 200 mg/L phenol and were incubated for 28 to 30 days at 37°C. The residual phenol was then measured by GC analysis. Those cultures which contained <80% of the initial phenol concentration were scored as positive. The MPN values were then obtained from appropriate tables (APHA, 1976; Alexander, 1965a).

6.2 Results and Discussion

6.2.1 Kinetics of Phenol Degradation in Batch Cultures

The rate of substrate removal from batch cultures has been related to the concentrations of microorganisms and substrate in many biological systems by the following expression (Lawrence and McCarty, 1970; Tempest, 1970; Robinson and Tiedje, 1983):

$$\frac{dS}{dt} = \frac{kXS}{K_s+S} \tag{6.1}$$

where $\frac{dS}{dt}$ = overall substrate utilization rate, mass volume^{-1} time^{-1}

k = maximum rate of substrate utilization per unit weight of microorganisms (occurring at high substrate concentration), time^{-1} (also $k=\mu max/Y$, where μmax is the maximum specific growth rate and Y is the growth yield coefficient)

X = microbial mass concentration, mass volume^{-1}

S = substrate concentration surrounding the the microorganisms, mass volume^{-1}

K_s = half velocity coefficient, equal to the substrate concentration when $dS/dt =(1/2)k$, mass volume^{-1}.

The data in Figure 6.1 show the substrate depletion curves for the batch cultures which initially contained 43, 59, 84, 104, 128, 148, and 199 mg/L phenol. Each of these cultures required 10 to 12 days incubation before phenol degradation began. Once the degradation started, the rate of removal increased constantly until phenol could no longer be detected in the medium. Although not shown, similar substrate depletion curves were observed for all of the other cultures which initially contained between 300 and 700 mg/L phenol.

From the shape of the substrate depletion curves (Figure 6.1), it was apparent that these batch cultures did

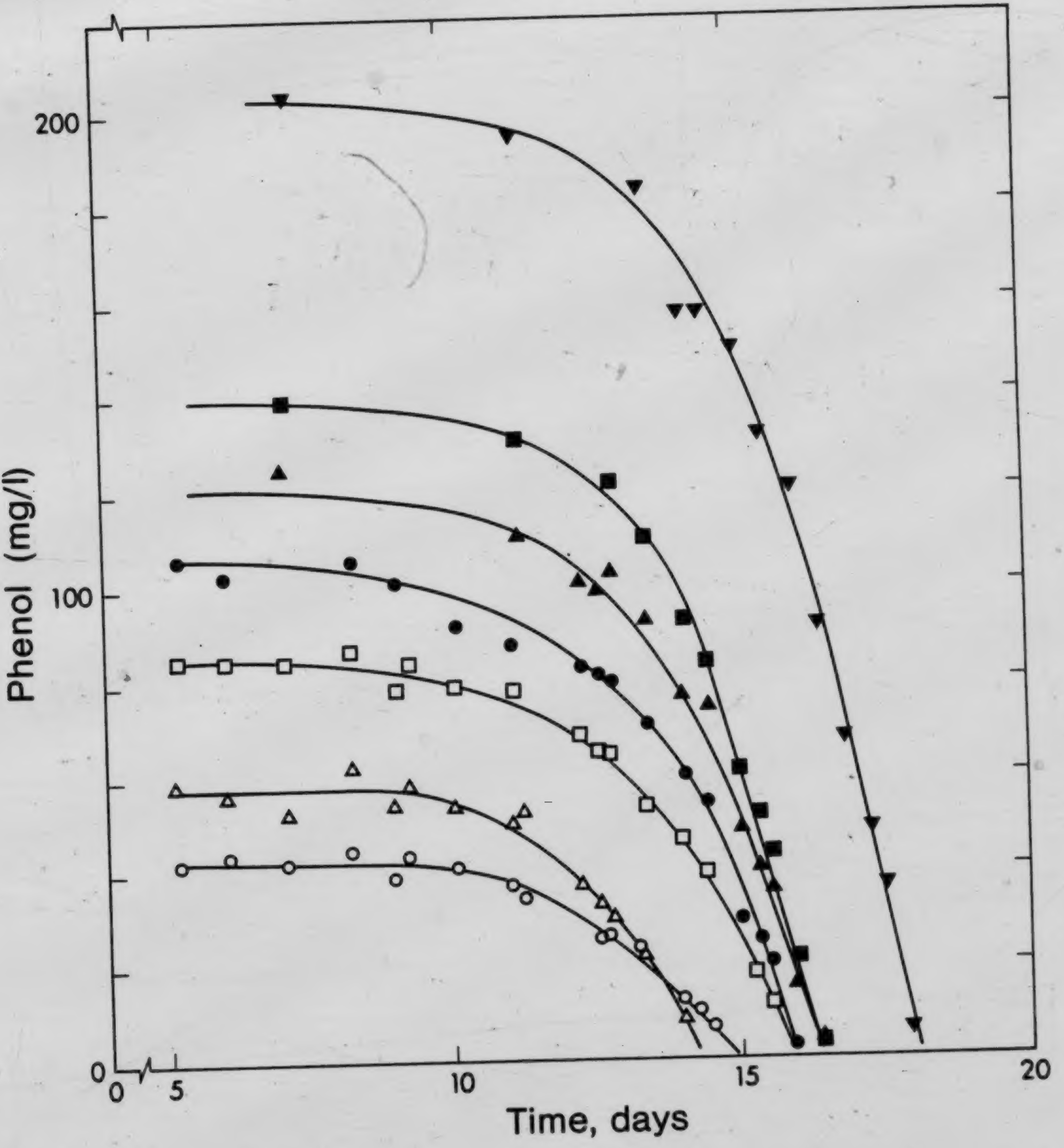


Figure 6.1 Selected substrate depletion curves from phenol-containing batch cultures. The initial phenol concentrations were 43, 59, 84, 104, 128, 148 and 199 mg/L.

not follow the Monod kinetic model which predicts a sigmoidal curve with the rate of removal decreasing as the substrate concentration approaches zero (Robinson and Tiedje, 1983). Such sigmoidal curves have been reported for glucose metabolism by aerobic, heterogeneous bacterial cultures (Gates and Marlar, 1968); nitrite consumption by *Nitrobacter winogradskyi* (Corman and Pave, 1983); and H_2 consumption by *Desulfovibrio* sp. (Robinson and Tiedje, 1983). Because of the shape of the phenol depletion curves, application of the Monod model was judged to be inappropriate for the substrate removal for the batch cultures.

However, the batch culture data do provide observations on the relationship between substrate removal rate (dS/dt) and the initial substrate concentration (S). This relationship was explored by determining the maximium phenol degradation rate for each culure. This was calculated from the substrate depletion curve using a minimum of five data points to define the line. A regression analysis was done on the last five observations for each batch culture and the standard error of estimate was calculated using the following expression:

$$\text{standard error of estimate} = (\Sigma(C-C_{est})^2/(n-2))^{1/2} \qquad (6.2)$$

where C = measured phenol concentration
C_{est} = estimated phenol concentration based on the least squares equation.
n = number of data points considered.

The sixth last data point was then added to the group of five points and the calculations were repeated. Addition of data points was continued until the standard error of estimate for the regression equation reached a minimum. The corresponding n data points defined the regression equation and, therefore, the value of dS/dt. The values of n ranged from 5 to 9.

The data in Table 6.1 show that higher initial phenol concentrations lead to larger final substrate removal rates (dS/dt). These ranged from 11.8 mg/L/day when the initial phenol concentration was 43 mg/L to near 100 mg/L/day when phenol was initially at 691 mg/L. In the range of 43 mg/L to 199 mg/L, the loss of phenol followed the first order reaction equation (dS/dt)=kS. As shown in Figure 6.2, a plot of (dS/dt) vs S over this range gave a straight line as predicted for a first order reaction. Beyond initial phenol concentrations of 200 mg/L, these data deviated from linearity and the first order model.

Typically the substrate removal rates in biological wastewater treatment processes are related to the amount of active biomass present. However, it is difficult to obtain a reasonable measure of the active microbial mass concentration. In this experiment, the volatile nonfiltrable residue (volatile suspended solids) concentration was evaluated as an approximation of X (Lawrence and McCarty, 1970). The mean volatile nonfiltrable residue and standard deviation of six replicates of the sludge used as inoculum were 2 620 and 12.6 mg/L, respectively. Since a 5-mL

Table 6.1 Summary of phenol removal rates and volatile nonfiltrable residue concentrations in batch cultures containing various amounts of phenol. Methane production was observed in all cultures.

Initial phenol concentration(1) (mg/L)	Maximum removal rate (dS/dt) (mg/L/day)	Final concentration volatile nonfiltrable residues(2) (X) (mg/L)
0 (Controls)	NA(3)	1 000
	NA	988
	NA	1 012
	NA	1 025
43	11.8	1 000
59	15.8	1 025
64	14.2	1 038
84	19.1	988
100	27.4	1 100
104	30.2	1 075
128	32.4	1 075
148	41.5	1 050
199	54.5	962
300	60.0	1 000
409	56.2	950
482	65.3	938
547	79.4	1 050
563	79.4	950
590	101	988
691	99.4	950

(1) Mean values determined by GC analyses prior to the start of phenol biodegradation

(2) Initial mean volatile nonfiltrable residue concentration was 1 310 mg/L

(3) NA = not applicable

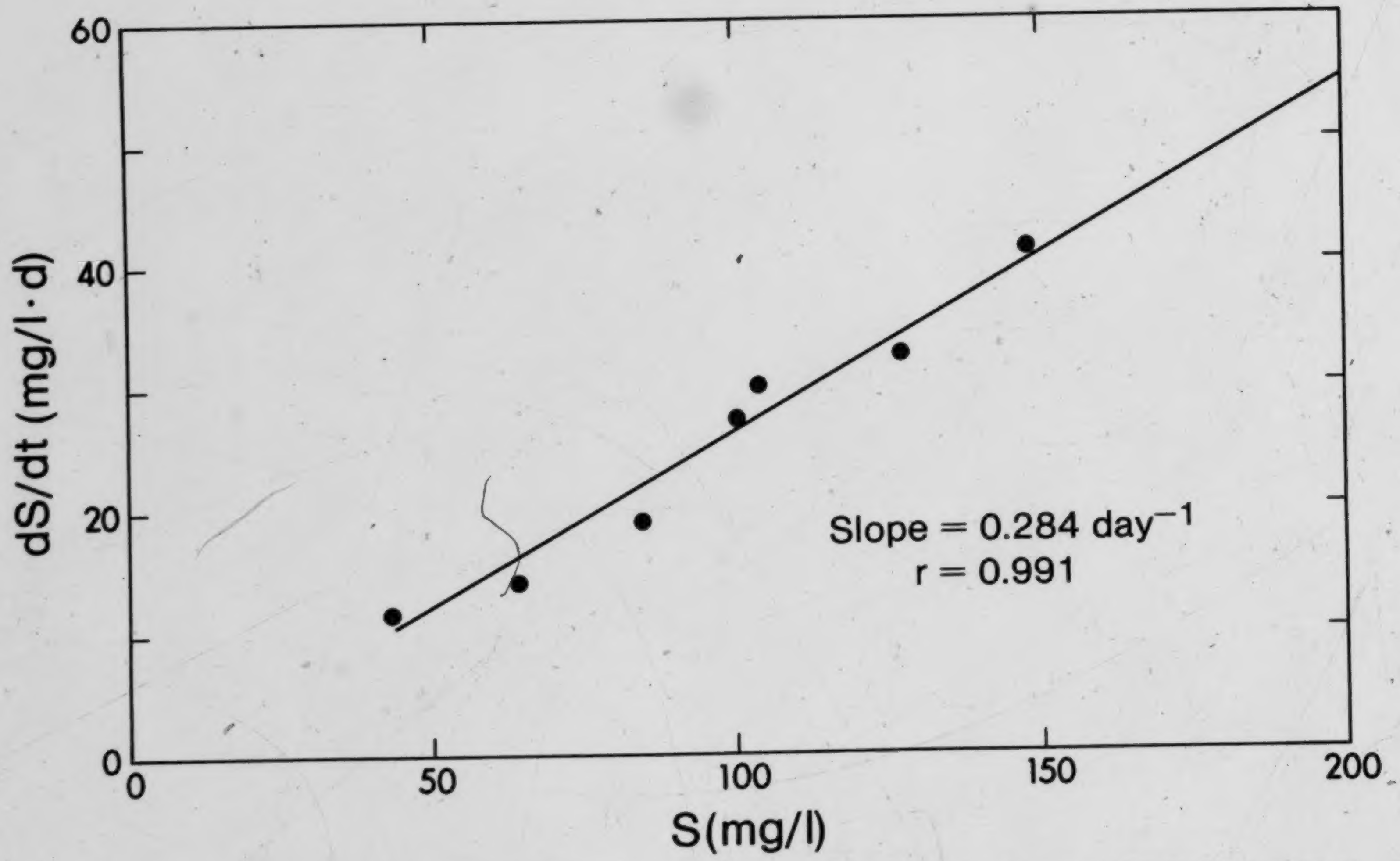


Figure 8.2 Maximum batch rate of phenol removal as a function of initial substrate concentration. Over the range of 43 mg/L to 199 mg/L, these data follow the first order reaction equation (dS/dt)=kS.

inoculum was added to 5 mL of medium, the concentration in the culture bottle at the time of inoculation was 1 310 mg/L. After the batch cultures were terminated, volatile nonfiltrable residue analyses were done on each of the controls, which were devoid of phenol, and each of the phenol-containing methanogenic cultures. These data are summarized in Table 6.1. The mean concentration in the control cultures was 1 010 mg/L. The mean value for the 16 phenol-containing cultures was also 1 010 mg/L. An analysis of variance and the Duncan's multiple range test showed that the latter two means were significantly less than 1 310 mg/L ($P>0.05$) indicating that the anerobic fermentation had reduced the volatile nonfiltrable residue content of the inoculum by 22.9%. Since it is likely that most of the fermentation of the organic solids occurred prior to the start of phenol degradation, the volatile nonfiltrable residue concentrations measured at the end of the incubation period were used as an estimate of X.

Although some of the measured volatile nonfiltrable residue concentrations of the phenol-containing cultures were significantly greater than values found in the controls, there was no correlation between the amounts phenol and the residue concentrations in the cultures. Thus increases in X resulting from phenol metabolism could not be detected.

The rates of phenol removal (dS/dt) can be compared with the volatile nonfiltrable residue concentrations (X) in

Table 6.1. Despite the significant rise in dS/dt at increasing S values, the X values remain relatively constant. Because one would expect higher values of phenol-degrading biomass to occur in the cultures exposed the higher phenol concentrations (S) and exhibiting higher phenol removal rates (dS/dt), volatile nonfiltrable residue is clearly inadequate as an indicator of phenol-degrading biomass.

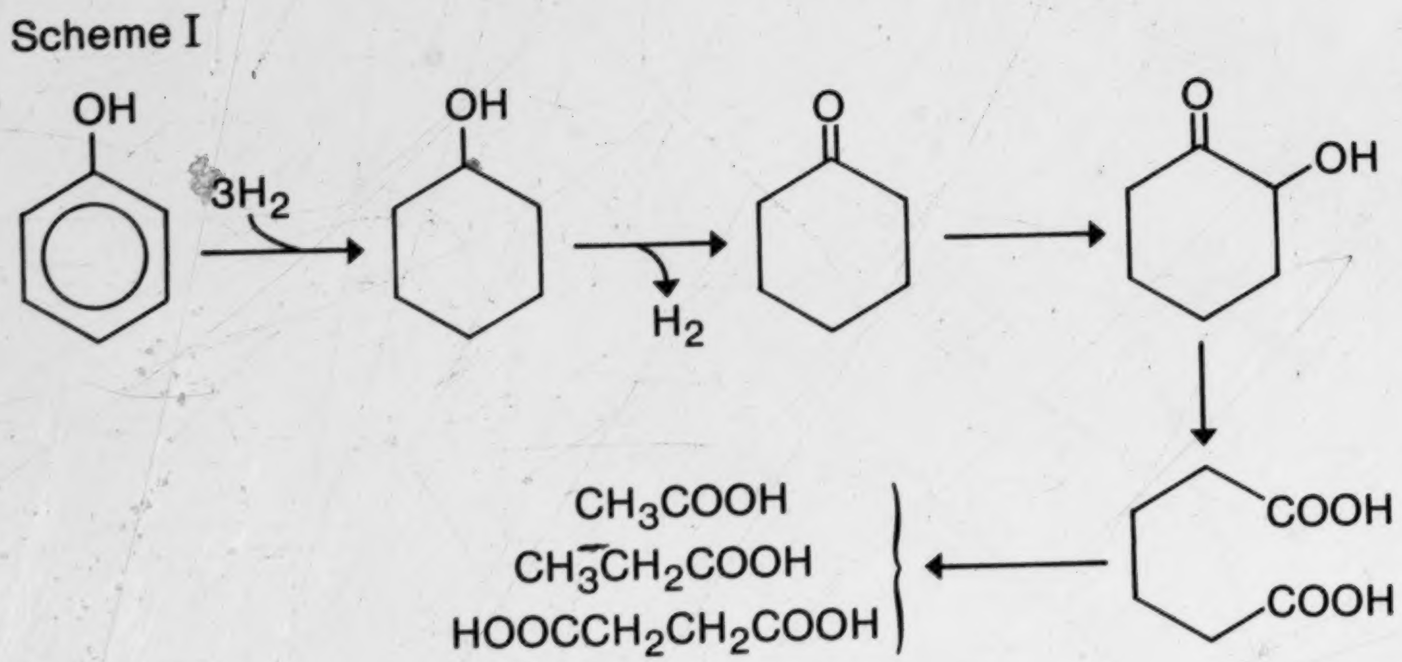
Consequently, although it is possible to convert the substrate removal rates, dS/dt, (mg/L/day) to a specific substrate removal rates, $dS/dt \bullet 1/X$, (day^{-1}) the calculated values would not provide meaningful results. Because X does not vary significantly, the specific substrate removal rates would follow the pattern experimentally observed for dS/dt, but the specific rates calculated in this manner would not truly indicate the amount of substrate removed per unit of active biomass. As a result, a specific utilization rate calculated in this manner does not contribute to an understanding of the microbial process dynamics beyond what can be learned from the direct observation that dS/dt is a first order function of S (when S varies between 43 and 199 mg/L phenol).

The observations from this series of batch cultures prompted further experiments to determine whether hydrogen was limiting at the onset of phenol degradation and to evaluate a MPN technique to provide a specfic measure of the active biomass in phenol-degrading cultures. An understanding of the factors which limit the beginning of phenol degradation could lead to changes in culture conditions to ultimately shorten the start-up time for anaerobic bioreactors treating phenolic wastewaters.

6.2.2 Propionate-supplemented batch cultures

Each substrate depletion curve (Figure 6.1) exhibited a phenol degradation rate which was slow at the start but continuously increased until the substrate had been depleted. Attempts were made to determine why the initial rates were low and to test the hypothesis that, at the start of phenol degradation, hydrogen was limiting.

Scheme I (Figure 6.3) shows the intermediates detected in a methanogenic consortium fermenting $[U-^{14}C]$ phenol (Balba and Evans, 1980) and illustrates that molecular hydrogen is thought to be involved during the initial reduction of the aromatic ring. One of the intermediates is propionic acid which is metabolized to 1 mole acetic acid and 3 moles H_2 as shown in Scheme II (Boone and Bryant, 1980; Wuhrmann 1982). Thus, once the degradation of phenol has started, the 3 moles of H_2 produced from 1 mole propionate would satisfy the H_2 requirements to reduce 1 mole of phenol to cyclohexanol (Scheme I). Ferry and Wolfe (1976) have detected H_2 in benzoate-degrading anaerobic mixed cultures and therefore it is likely to be present in phenol-degrading cultures.


Scheme I
OH
3H2
OH
H2
O
O
OH
COOH
COOH
CH3COOH
CH3CH2COOH
HOOCCH2CH2COOH

Scheme II

$$2H_2O + CH_3CH_2COOH \longrightarrow CH_3COOH + CO_2 + 3H_2$$

Figure 8.3 Degradative pathway for phenol in a methanogenic consortium. Scheme I Postulated pathway and identified intermediates of phenol degradation (After Balba and Evans, 1980). Scheme II End products of propionic acid degradation.

Molecular hydrogen is produced from the fermentation of many organic compounds normally present in the sludge inoculum (Gray and Gest, 1965). However, it is unlikely that much hydrogen from these sources would be involved with phenol reduction. Based on observed methane production, the fermentation of non-phenolic substrates has essentially stopped prior to the start of phenol degradation. For example, Figure 5.7 shows that in cultures which were supplemented with NBG, methane production in the control cultures had nearly reached a plateau before there was evidence of phenol degradation.

Propionate was initially chosen as a source of molecular hydrogen for two reasons. First, the propionate-degrading organisms are inhibited by low concentrations of H_2 (Boone and Bryant, 1980) and it was thought that the addition of H_2 directly might upset the anaerobic process. Second, Sleat and Robinson (1983) have found that prior enrichment of methanogenic consortia on propionate (as well as acetate, butyrate, or valerate etc.) decreased the acclimation time required for benzoate degradation.

The loss of phenol from selected propionate-supplemented cultures is shown in Figure 6.4. Sodium propionate added to give a final concentration of 192 mg/L, provided a potential production of 2 moles of H_2 for each mole of phenol initially present in the batch culture. This amount would satisfy the overall H_2 requirement leading to the formation of cyclohexanone (Scheme I, Figure 6.3). The

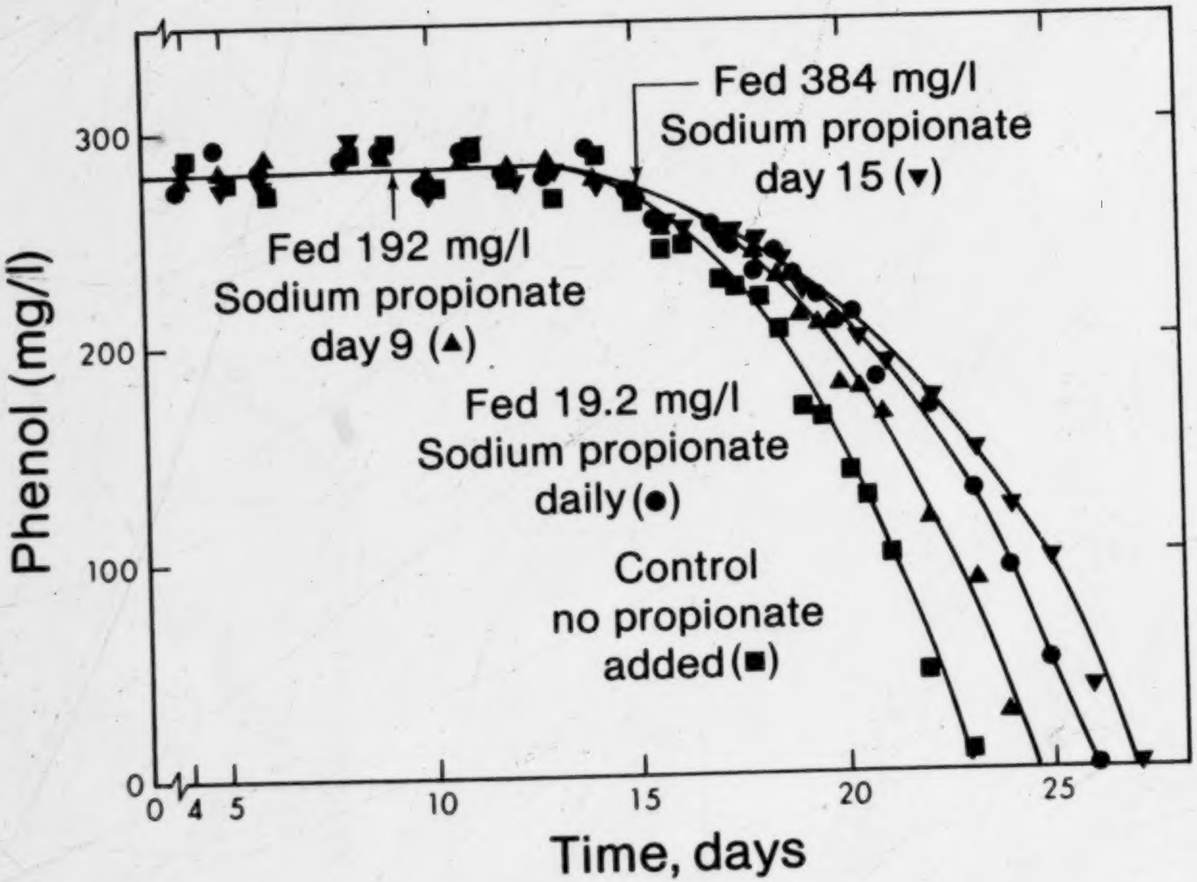


Figure 8.4 Substrate depletion from phenol-containing batch cultures supplemented with sodium propionate at various times.

sodium propionate dose of 284 mg/L would potentially yield double the required H_2 for this reduction. Since the overall consortium would compete for H_2 (i.e. methane bacteria, sulfate reducers and presumably phenol-degraders) sufficient propionate was added to provide H_2 in excess of that needed for phenol reduction.

Phenol degradation was retarded rather than enhanced in five of the seven sets of cultures supplemented with propionate. In the other two sets, those which received single propionate doses at the time of inoculation, substrate depletion curves were not significantly different ($P<0.05$) from the control cultures which received no propionate. These data have not been included in Figure 6.4. Propionate addition at time zero produced methane concentrations in excess of those in the controls when analyzed after four days of incubation (Figure 6.5). Thus, the inoculum contained an active population of propionate-degrading organisms. After the initial burst of methane formation, the concentrations of methane in the propionate-supplemented cultures remained parallel to that in the control culture which contained phenol but was devoid of added propionate. It is likely that there was no residual propionate in the cultures when phenol degradation started. Therefore, the rate of substrate loss was unaffected.

The daily addition of small doses of sodium propionate (equivalent to 19.2 mg/L final concentration/day) retarded the start of phenol degradation (Figure 6.4) but it did not

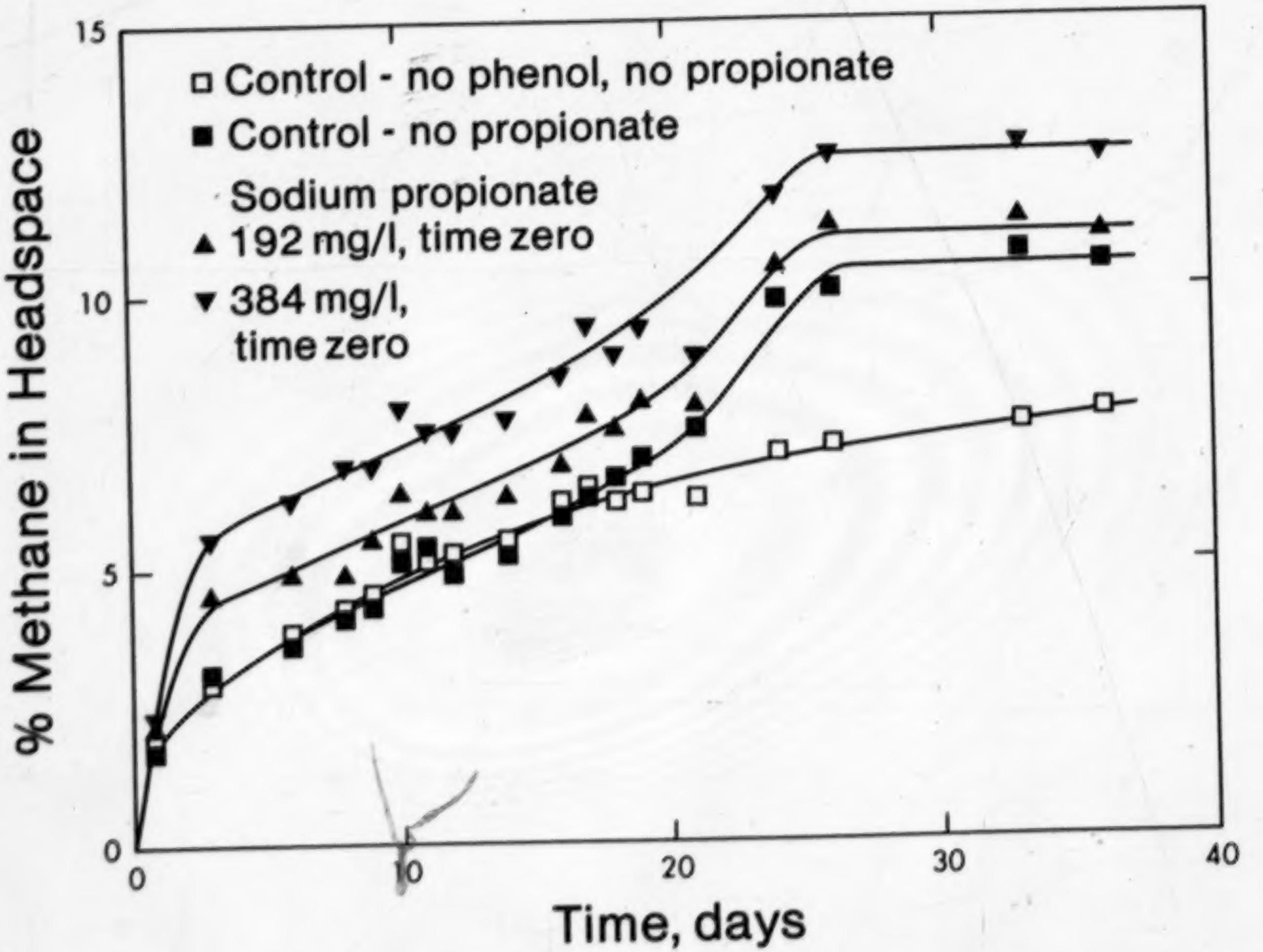


Figure 6.5 Methane production in batch cultures supplemented with sodium propionate at time zero.

appear to decrease the rate of removal during the later stages of the batch fermentation.

Propionate supplementation on day 9 did not shorten the acclimation period by providing H_2 just prior to the start of phenol degradation. Figure 6.4 shows that when sodium propionate was added at a concentration of 192 mg/L, phenol removal was slower than in the non-propionate containing controls. Similar results were obtained (but are not shown) when 384 mg/L sodium propionate was added on day 9. The addition of propionate on day 15 - the time at which phenol loss was first detected - had the greastest inhibitory effect on phenol removal (Figure 6.4). Again the addition of 192 or 384 mg/L sodium propionate gave essentially the same result.

The methane production data from the cultures supplemented on day 9 (which has not been included) showed a burst of methane by day 12 which was attributable to propionate rather than phenol-degradation. This was similar to the trend observed when the cultures were supplemented at time zero (Figure 6.5).

Methane evolution from the cultures which were supplemented on day 15 (Figure 6.6) did not show the same burst as seen in the other cultures. In this case, the methane concentrations remained the same as those in the phenol-containing control cultures until after day 24 (i.e. there was no enhanced methane over that 9-day period). This was in contrast to bursts occurring within four days and three days

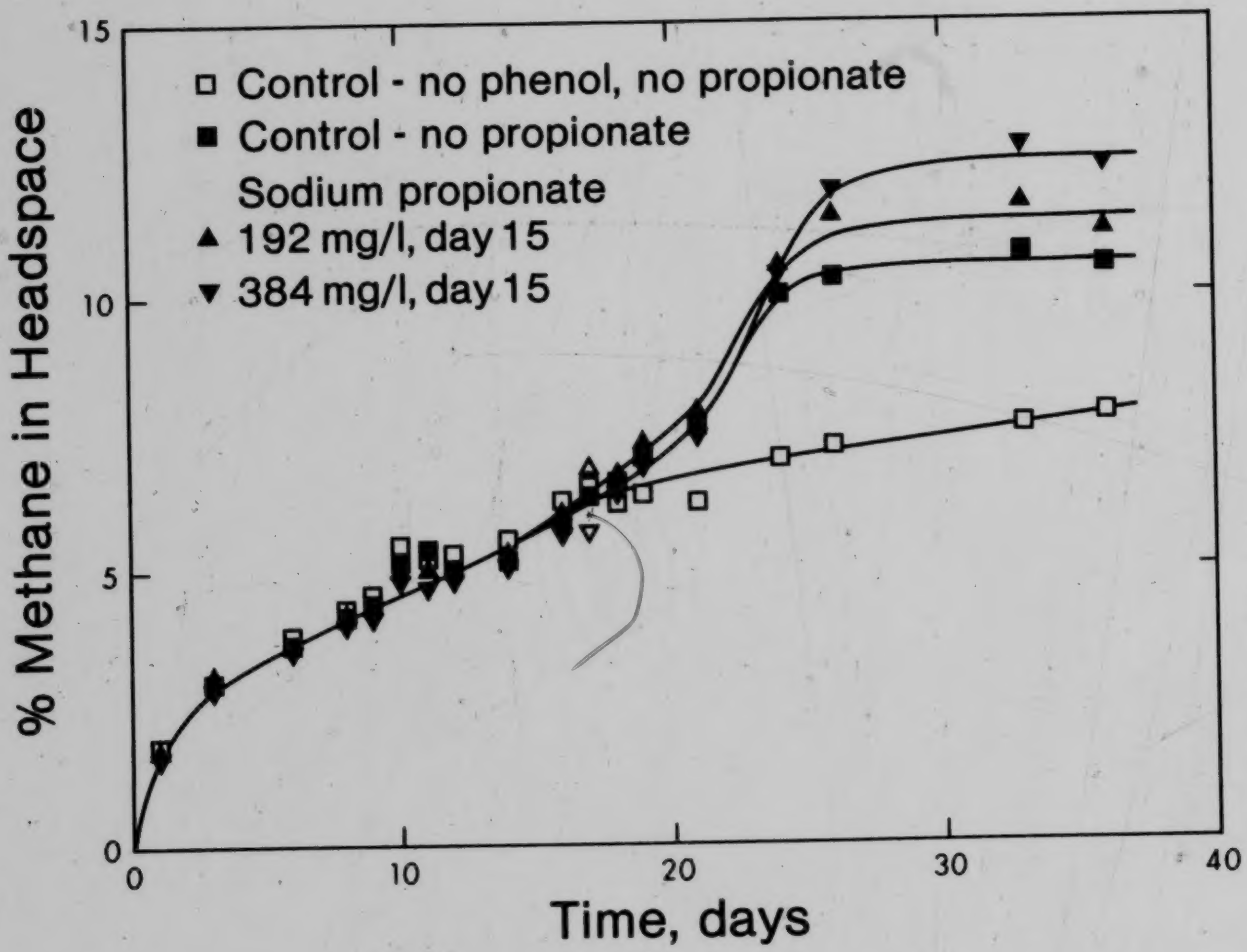


Figure 6.6 Methane production in batch cultures supplemented with sodium propionate on day 15.

in the time zero and day 9 supplemented cultures, respectively. During this 9-day period, the 384 mg/L supplemented culture reduced the phenol concentration by approximately 140 mg/L (Figure 6.4) while the non-supplemented culture reduced the phenol concentration by nearly 260 mg/L. Thus the addition of propionate to batch cultures at start of phenol degradation caused both inhibition of phenol removal and inhibition of the conversion of propionate to methane.

Exposure of these batch cultures to propionate prior to the start of phenol degradation (i.e. the time zero, day 9, and daily supplementation) did not enrich an active phenol-degrading population. Sleat and Robinson (1980) reported that prior establishment of methanogenic cultures on propionate, decreased the adaptation time for benzoate degradation. Although they did not specify the propionate enrichment period required for this effect, the relatively short exposure time and small propionate doses used in the present project were not sufficient to provide the enhanced phenol degradation.

The data clearly demonstrates that the addition of propionate as a hydrogen source did not overcome any limitations to phenol degradation. It also served to illustrate the complexity of the microbial activities in the mixed population. To date, anaerobic microorganisms which are able to cleave the aromatic ring (i.e. phenol- and/or benzoate-degraders) have not been isolated from methanogenic consortia. Some authors (McInerney et al., 1979; Zeikus

1979) speculate that these are members of the hydrogen producing acetogenic group (section 2.2) which also includes the propionate degrading bacteria. The observations from the propionate supplemented phenol-containing cultures are consistent with these speculations. The data indicate if propionate was present when phenol degradation was occurring, as was the case for the daily feed cultures and the day 15 supplemented cultures, phenol degradation was inhibited. This suggests that propionate is the preferred substrate, but as seen from methane data of the day 15 supplemented cultures (Figure 6.6) phenol-degrading activity also retards the conversion of propionate to methane. As yet, it is not known whether these changes in the microbial activities are due to the regulation of metabolic processes within a single phenol-degrading species or due to the interaction or competition between species within the mixed population.

6.2.3 Hydrogen-supplemented Batch Cultures

To further investigate the role that molecular hydrogen plays in the control of the degradation rate of phenol, another series of phenol-containing batch cultures was inoculated. In this case, H_2 was injected directly into the cultures at times that matched the propionate-supplementation experiment. The volumes of H_2 were measured at ambient temperature and pressure and each mL added corresponded to approximately 0.04 mmole. Thus a dose of

1.5 mL was sufficient to satisfy the amount of H_2 required to reduce all the phenol in the 10 mL culture (300 mg/L, initially) to cyclohexanone (Scheme I, Figure 6.4) and was equivalent to the addition of 192 mg/L sodium propionate. The maximum volume injected was 3.0 mL which gave double the amount of H_2 required for the reduction of phenol (equivalent to 384 mg/L sodium propionate). Upon addition, this volume gave approximately 6% H_2 in the culture bottle headspace gas which was well below the 10% concentration that Sleat and Robinson (1983) found to completely inhibit $^{14}CH_4$ production from [ring-U-^{14}C] benzoate in established enrichments.

At the time of inoculation, doses of 0.5 mL and 1.5 mL H_2 were added to two sets of cultures. The smaller volume was not sufficient to give detectable amounts of methane (above controls) until day 8, whereas the cultures which received the larger volume showed elevated methane concentrations within one day. Another series of cultures received daily additions of 0.15 mL H_2 so that by day 10, each culture had received enough H_2 to reduce all of the phenol. Only one of these supplementations altered the degradation rate of phenol as shown in Figure 6.7. The addition of 0.5 mL H_2 at time zero, hastened the loss of phenol from the cultures.

On day 9, aliquots of 1.5 and 3.0 mL H_2 were added to two sets of cultures. The addition of the larger volume stimulated the phenol degradation rate (Figure 6.7), which

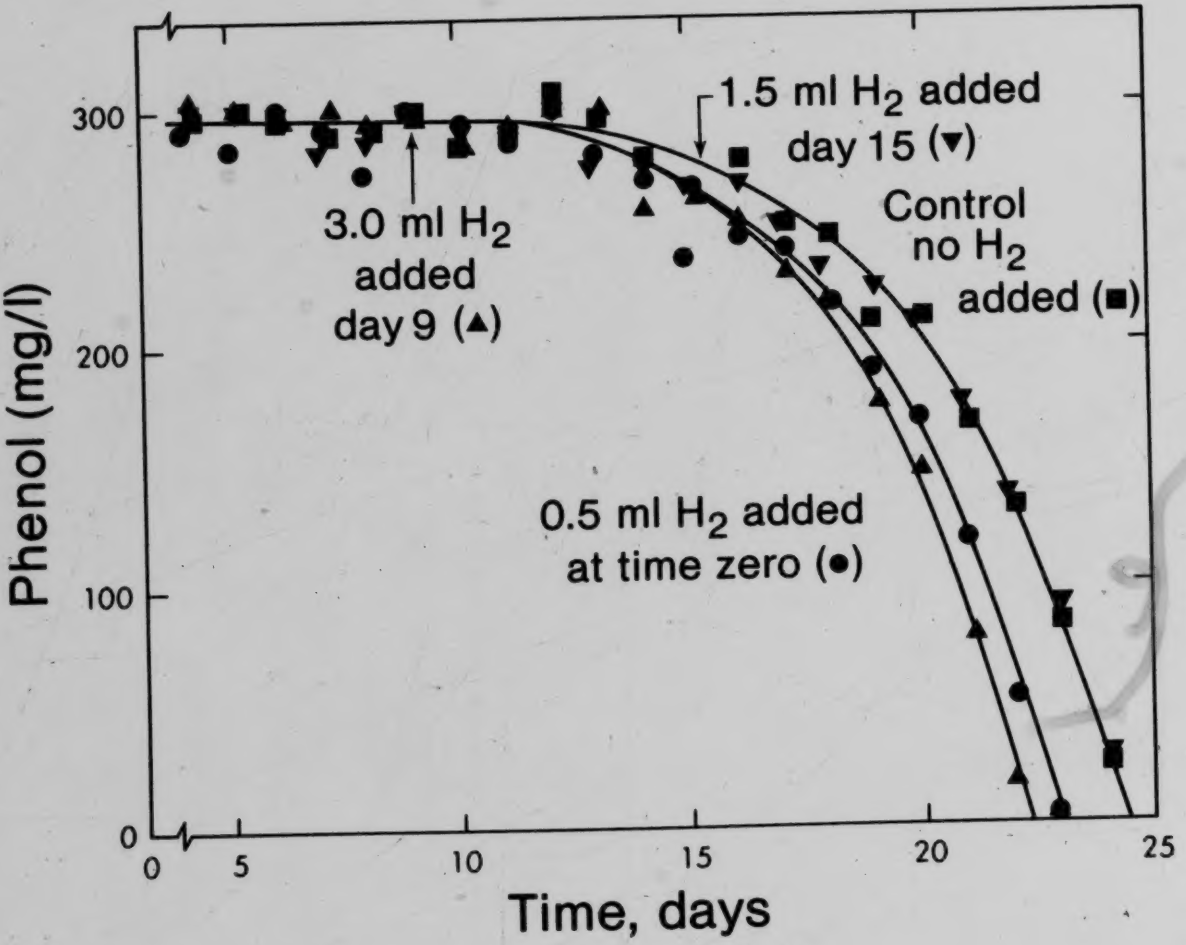


Figure 6.7 Substrate depletion from phenol-containing batch cultures supplemented with hydrogen at various times.

by day 16, was significantly greater than that of the non-supplemented control cultures. The smaller volume did not alter the rate of phenol depletion.

Phenol loss was detected on day 15 in those cultures which had not been supplemented with H_2 and volumes of 0.5, 1.5 and 3.0 mL were added to three sets of cultures at that time. The substrate depletion curve for the cultures which received 3 mL H_2 is shown in Figure 6.7 and is typical of the results obtained from the other two sets of cultures supplemented on day 15. That is, the rates of phenol removal from these cultures were indistinguishable from that in the non-supplemented controls.

The results from this experiment strongly suggest that H_2 is not the limiting factor at the start of phenol degradation in batch cultures since the supplementation on day 15 did not enhance the rate of removal. However, it appears that molecular hydrogen does play some role since in two cases its addition did stimulate phenol degradation. In these cases, the effect of dosage was unclear since the smaller volume added at time zero (0.5 mL) and the larger volume added on day 9 (3.0 mL) were the only concentrations which increased the degradation rates. At the time phenol breakdown started, it seems unlikely that any of the 0.5 mL H_2 added at time zero would remain since methanogens actively convert H_2 to methane. If these results are suggesting that the presence of very small concentrations of H_2 are stimulatory to phenol degradation, it is more likely that the daily fed cultures or the day 9, 1.5-mL supplemented cultures would have given enhanced phenol degradation rather than the 3.0-mL supplemented day 9 culture.

The role of H_2 and the source of hydrogen for the reduction of the aromatic ring were not pursued further in this project. These present interesting experimental challenges that will likely improve our understanding of the biochemical activities that may control the rate of phenol degradation in anaerobic environments under steady state conditions. However, the aim of the propionate- and hydrogen-supplementation experiments was to determine whether H_2 was the limiting factor as phenol degradation began. The experimental results indicate that H_2 did not play a major role at that time.

6.2.4 MPN Results

Although many investigators have tested the abilities of various sludges, muds and sediments to yield phenolic degrading methanogenic consortia, none has reported estimates of the numbers of organisms in these samples which are capable of phenolic breakdown. From enrichment cultures, Tsai and Jones (1975) have isolated rumen bacteria which degrade 1,3,5-trihydroxybenzene (phloroglucinol). However they could not isolate these directly from the rumen fluid suggesting that these organisms were present in very small numbers.

The numbers of phenol-degrading organisms present in the domestic sludge were of interest for two reasons. Firstly, the numbers of active microorganisms, rather than H_2 availability, could be limiting at the time phenol degradation began. For example, earlier work with batch cultures (section 5.2.4) suggested that the phenolic-degrading organisms are numerically a minor portion of the microbial population in the sludge. If this is true, relatively small increases in their numbers within a batch culture would cause the increasing substrate depletion rates as observed in Figure 6.1. Secondly, the ability to estimate the numbers of phenol-degrading organisms would allow more specific monitoring of a phenol-degrading consortium than presently available with the volatile nonfiltrable residue measurement which was shown to be inadequate in section 6.2.1.

The MPN technique is a statistical estimate of the density of a microbial population which is determined through the use of dilution and multiple tube inoculation. Table 6.2 summarizes a wide number of applications for which the MPN technique is used. In general, when the organism(s) of interest cannot be readily plated on selective or differential solid medium to facilitate counting, the MPN method is used. This method is also applicable for the enumeration of microorganisms with specific metabolic capabilities. Examples, such as heterotrophic microorganisms, methane producers and petroleum degraders, are given in Table 6.2.

Table 6.2 Survey of the applications of the MPN technique.

Microorganisms or metabolic activity	Reference	Comments
Algae	Clark and Durrell (1965)	Tubes which contain green, blue-green or other coloured growth are examined microscopically to detect algal growth and scored accordingly.
Azotobacter	Clark (1965a)	Positive tubes show growth as a skin or pellicle on the surface of medium which is free of a fixed nitrogen source.
Beggiatoa	Strohl and Larkin (1978)	Positive tubes contained macroscopic "fluff ball" tufts of colonies characteristic of these filamentous bacteria.
Coliforms	APHA (1980)	Gas and turbidity produced from lactose at 35°C (total coliforms) and 42.5°C (fecal coliforms).
Denitrifying bacteria	Alexander (1965b)	Conversion of NO_3^- to N_2 causes gas evolution and pH increase in positive tubes.
Enteric viruses	APHA (1980)	Cytopathic effects in cell cultures or characteristic symptoms in test mice are considered as positive results.
Heterotrophic microorganisms	Lehmicke et al. (1979)	Individual ^{14}C-labelled substrates (including glucose, benzoic acid, 2,4-D and Dicamba) added to dilutions of natural waters. Release of >1% of the added label as $^{14}CO_2$ was considered positive.
Methane producers	Siebert and Hattingh (1967)	Appropriate substrate (eg. acetate, formate) added to dilutions of sludge. Tubes which produced methane as determined by GC, were positive.

Table 6.2 (cont.)

Microorganisms or metabolic activity	Reference	Comments
Nitrifying bacteria	Alexander and Clark (1965)	Tubes positive for *Nitrosomonas* produce NO_2^- from NH_4^+ and those positive for *Nitrobacter* produce NO_3^- from NO_2^-.
Petroleum degrading microbes	Mills et al. (1978)	Visible turbidity, which was correlated to protein increase and degradation of n-alkanes, was scored positive in medium containing 1% crude oil.
Rhizobia	Clark (1965b)	Aliquots of diluted sample poured onto appropriate legume seed in sterile sand. After satisfactory growth under greenhouse conditions plants with root modules are scored as positive.
Sulfate-reducing bacteria	Obuekwe et al. (1983)	Production of $S^=$ forms FeS on iron nails in culture tube. Blackening of nails scored as positive.

Colwell (1979) notes that the MPN method can only be useful if the organisms being enumerated cause a characteristic or readily recognizable transformation in the medium into which they have been inoculated. The transformation may be a chemical change in the medium or the appearance of turbid or specifically recognizable microbial growth in the culture medium. The MPN culture is scored "positive" when the transformation is observed. The comments in Table 6.2 indicate the wide range of microbial activities which have been used as a basis to score MPN cultures.

Colwell (1979) also emphasized that a single cell must be capable of initiating growth (and therefore bringing about the recognizable transformation) in the medium used. This situation is unlikely in the case for anaerobic phenol-degrading populations since interspecies hydrogen transfer is an important process in such environments (McInerney et al., 1979; Zeikus, 1979) and H_2 is required for phenol degradation (Figure 6.3). For this reason, the term phenol-degrading units (PDU), rather than phenol-degrading organisms, has been used.

The presence of a PDU in the MPN growth medium was detected by a decrease in the phenol concentration after a suitable incubation period. Previous work with batch cultures showed that at an initial concentration of 200 mg/L, phenol was completely removed within approximately 20 days. Therefore, in the MPN procedure, the cultures were routinely allowed to incubate for 30 days prior to phenol analyses to ensure adequate time for phenol degradation. Since there were some variations in the GC analyses, cultures which gave <80% of the original phenol concentration after this time were scored as positive while all others were scored as negative.

The data in Table 6.3 summarize the results of the four MPN trials. The number of PDU detected by the MPN method was suprisingly low considering the total anaerobic population in domestic sludge is typically 10^{10} to 10^{12} bacteria/100 mL. The extreme MPN values observed in the anaerobic domestic sludge (trials 1 and 3) can be compared by the method of Cochran (1950). For the 10-fold dilution series which give $MPN_1=80$ (based on a 3-tube test) and $MPN_2=23$ (based on a 5-tube test) the significance of the difference between these values is given by:

$$Z = (\log MPN_1 - \log MPN_2)/(0.58(1/3+1/5)^{1/2}) \qquad (6.3)$$

Calculating $Z=1.28$ for these data and referring to normal probability tables, show that there is no significant difference between these MPN values ($P>0.05$).

Although the MPN data are quite reproducible (e.g. trials 1, 2 and 3), they appear to underestimate the true number of PDU as illustrated by considerations of expected cell yields from phenol consumption and from required phenol transport rates in batch cultures. In order to proceed with these considerations, data on bacterial cell size and composition, numbers of acid-formers in anaerobic sludge and typical bacterial substrate uptake rates are required. These

Table 6.2 Summary of MPN results from attempts to enumerate PDU in anaerobic sludges. All MPN cultures incubated at 37°C for 28-30 days unless otherwise stated.

Trial No.	Sample Source	Inoculation volumes(mL)	No. of positives(1)	PDU MPN/100 mL
1(2)	Domestic anaerobic sludge.	0.5 0.05 0.0005	1 0 0	80
2a(2)	Domestic anaerobic sludge prior to exposure to phenol.	10 1 0.1	3 1 0	43
2b(2)	Domestic anaerobic sludge after exposure to phenol.(3)	10 1 0.1	3 1 0	43
3(4)	Domestic anaerobic sludge.	10 1 0.1	5 0 0	23
4(4)	Anaerobic sludge blanket reactor treating pulping wastewater.(5)	1 0.1 0.01	2 (5)(6) 4 (4) 0 (1)	150 (1 700)(6)

(1) <80% of the phenol remaining in the MPN culture medium.
(2) 3-tube MPN.
(3) Contents from a 50 mL batch culture after depletion of 200 mg/L phenol from medium.
(4) 5-tube MPN.
(5) From Dr. J.F. Ferguson, University of Washington, Seattle, Wa. (Woods et al., 1983).
(6) After 44 days incubation at 37°C.

are summarized in Table 6.4.

Trial 2 was undertaken to estimate the increase in PDU in a batch culture. MPN analyses were done on the sludge used as the inoculum and on the culture after the phenol had been consumed. The experimental results were compared to possible cell yield estimates. The inoculum had 43 PDU/100 mL (trial 2a, Table 6.3). Since the culture contained 45 mL sludge in a total volume of 50 mL, it contained 0.45 x 43 PDU = 19 PDU or 38 PDU/100 mL. The total amount of phenol in this culture was 200 mg/L x 0.05 L = 10 mg. Since phenol is 77% C, and 10% of the substrate-C can be expected to be converted to biomass, the amount of C incorporated into cells = 0.77 x 0.1 x 10 mg = 0.77 mg or 770 µg.

The dry weight of a typical bacterial cell is 30% of its total weight or 0.3×10^{-6} µg. Therefore the number of cells expected from the incorporation of 770 µg of phenol-C is (770 µg)/(0.3×10^{-6} µg/cell) = 2.6×10^{9} cells.

The proportional distribution of phenol-C among the various microbes within the population of the consortium is unknown. Three cases are suggested.

1. The phenol-degrading acid-formers do not incorporate any of the phenol-C. Therefore no increase in the number of PDU would be expected.
2. The phenol-C is distributed among the cells in accordance with their relative proportions at the time of inoculation. Initially there would be 10^{9} acid-

Table 6.4 Literature values for parameters required to consider the validity of the PDU MPN values.

Parameter	Literature values	Value used	References
Percent carbon converted to biomass under anaerobic conditions	10-20%	10%	McCarty (1964) Kirsch and Sykes (1971)
Wet weight of a bacterial cell	10^{-12}g	10^{-6}µg	Brock (1979)
Water content of a bacterial cell	70%	70%	Stanier et al. (1970)
Number of acid-formers in anaerobic sludge	$6 x 10^{8}$ - $1.5 x 10^{10}$/mL	10^{9}/mL	Toerien et al. (1967)
Initial rate of thiodigalactoside (TDG) uptake by *E. coli*	50 µmole/min/g	50 µmole/min/g	Kepes (1970)

formers/mL x 45 mL = 4.5×10^{10} in the culture bottle. The ratio of PDU to total acid-formers would be $19/(4.5\times10^{10})$ or 4.2×10^{-10}. Thus the expected number of PDU formed at the expense of the phenol-C is $(4.2\times10^{-10}) \times (2.6\times10^{9}) = 1.1$ PDU.

3. All of the phenol-C is converted to phenol-degrading acid-formers to give 2.6×10^{9} PDU at the end of the batch culture phase.

It is likely that the actual distribution of phenol-C lies between cases 2 and 3 since those organisms which can attack the substrate should have a selective advantage and therefore should incorporate a larger proportion of the C. However, since a microbial consortium is required to convert phenol to methane, it is anticipated that other acid-formers and the methane bacteria will also incorporate some of the substrate-C. The 3-tube MPN result on the dilution series made after 23 days incubation gave 43 PDU/100 mL (trial 2b). Thus, the MPN technique did not detect any increase in the number of PDU after the loss of phenol had been measured.

Assuming that the above MPN values were correct, then the number of PDU in a batch culture does not change during the time of phenol degradation. With this in mind, the rate of phenol uptake by the phenol-degrading acid-formers can be calculated and compared to another bacterial transport system. During the study of the kinetics of phenol removal in batch cultures (section 6.2.1), the final substrate degradation rate was 2.27 mg/L/h (54.5 mg/L/day) in the batch culture which initially received near 200 mg/L phenol (Table 6.1). Regardless of the number of types of microorganisms required to degrade phenol (i.e. the complexity of a PDU), presumably one type of bacterium must start the degradation process by transporting the phenol into its cell. Assuming that the MPN was 43 PDU/100 mL in the 10 mL culture, then 4.3 cells were transporting (2.27 mg/L/h)x(0.01L)/(94 mg phenol/mmole) = 0.00024 mmole/h or 0.24 μmole/h and each cell must transport (0.24 μmole/h)/4.3 = 0.056 μmole/h. Converting this value to units of μmole/min/g of dry cell weight (to match data provided in Table 6.4) gives: [(0.056μmole/h/cell)/(60 min/h)]/(0.3x10^{-12}g/cell) = 3.1x10^{9}μmole/min/g.

The typical initial substrate transport rate for TDG (a nonmetabolizable analog of lactose) into *E. coli* is 50 μmole/min/g. The preceding calculations show that the transport rate required to satisfy the observed phenol degradation rate is nearly eight orders of magnitude greater than the TDG uptake rate.

This large discrepancy strongly suggests that the number of cells available to take up phenol is unrealistically low and therefore the MPN results are suspect. There are probably two important reasons for these results to be lower than expected. One is the culture method used and the other is the application of the standard MPN tables. Both problems stem from the lack of understanding of the microorganisms and interactions among these organisms during the phenol degradation process.

The MPN culture conditions were derived from information gathered from batch cultures using sludge which was diluted 2 to 3 fold upon inoculation (e.g. section 5.1.1). Under these conditions, 200 mg/L phenol was not inhibitory and was readily degraded within 20 days. The same phenol concentration was used for the MPN method, the incubation time was extended to 30 days and the cultures were only required to remove 20% of the phenol in order to be scored as positive. The data from trial 4 (Table 6.3) illustrates two points about the method. First, although the method is quite crude, it was able to produce MPN data which could distinguish between domestic anaerobic sludge and a sludge which had been exposed to phenolic wastewaters for a long period of time. The latter sludge was extremely active and phenol degradation in batch cultures was observed after 3 days in contrast to typical times of near 2 weeks for the cultures containing domestic anaerobic sludge. The MPN cultures (trial 4) were inoculated from a batch culture which contained 3 mL of the active sludge in a total volume of 10 mL. The MPN score after 30 days incubation was 150 PDU/100 mL which means the original sludge contained 500 PDU/100 mL. (Qualitatively the solids content of this sludge was much higher than that typically observed in the domestic anaerobic sludge. However, since these were not quantitated, the MPN data were expressed on a per volume basis.)

The second aspect illustrated by the trial 4 data is that a longer incubation period produced a significantly higher MPN ($P>0.05$). The 30 day incubation gave 150 PDU/100 mL while the 44 day incubation gave 1 700 PDU/100 mL. The latter count indicates that the original sludge contained about 5 660 PDU/100 mL.

It is also possible that the initial phenol concentration used in the MPN tube was too high. When considering any bacterial population exposed to a specific concentration of an inhibitory compound (such as phenol), a certain fraction of the population will tolerate that concentration while the remainder will be sensitive. If for example, only 10% of the population of phenol degraders will tolerate 200 mg/L while 60% will tolerate 50 mg/L, then using the lower concentration of phenol in the MPN medium would produce high counts.

The application of the standard MPN tables may also be leading to low results. These tables have been derived on the assumption that a single cell can initiate growth in the medium. If phenol degradation requires interaction between species, then the basic assumption for use of the MPN tables is not met. In their characterization of an anaerobic consortium that converts 3-chlorobenzoate to methane and carbon dioxide, Shelton and Tiedje (1983) have found that a benzoate degrader exists in a syntrophic association with a methanogen. That is, both organisms are required for benzoate degradation.

Consider a simple case where 1 organism, A, can degrade phenol independently (i.e. 1 cell = 1 PDU) and 1 cell of the organism is in a 10 mL sample. When removing a 1 mL aliquot to transfer to another tube of medium (as is done in the MPN method) there is a 1 in 10 chance of transferring that PDU. In contrast, assume that 2 organisms, B and C, are required for phenol degradation (i.e. 1 cell B + 1 cell C = 1 PDU) and that 1 cell of each is randomly distributed in a single 10 mL sample. In a 1 mL aliquot, there is a 1 in 10 chance of withdrawing B and 1 in 10 chance of withdrawing C. However, the probability of withdrawing a PDU is $(1/10)^2$ or 1/100. Under the same conditions, if a 3-organism consortium is required to degrade phenol, then the probability of withdrawing a PDU in a 1 mL aliquot is 1/1000. Thus it is apparent that the MPN tables based on the first case (i.e. 1 in 10 chance) will underestimate the number of PDU when probabilities of 1/100 or less are possible.

These preliminary attempts at enumerating phenol degraders have not yielded counts which can be considered realistic in terms of other information known about this system. However, the MPN procedure does suggest that the number of PDU are likely not a major fraction of the total anaerobic population and it does indicate some of the problems associated with the application of the MPN method. For this procedure to produce better results, variations in media composition and incubation times must be evaluated further. Also, an understanding of the number of organisms which make up a PDU must be determined and with this knowledge, an appropriate set of MPN tables generated to satisfy the needs of the procedure. The former two tasks require in-depth microbiological studies of the phenol-degrading consortium, while the later task requires a return to the basic probability arguments of McCrady (1915) to produce new tables. This could only be done effectively if the number of organisms comprising a PDU and their relative abundance were know. Such studies were beyond the scope of the initial objectives of this thesis project and therefore, these aspects were not pursued.

7. FERMENTATION OF SYNTHETIC MIXTURES OF PHENOLICS IN BATCH CULTURES

Data presented in chapter 5 showed that in batch cultures, phenol at concentrations up to 500 mg/L was consistently fermented to methane after a suitable acclimation period. Similarly, p-cresol was degraded when present at levels up to 400 mg/L. The other eight alkyl phenolics were not extremely inhibitory to the anaerobic process until their concentrations reached ≥300 mg/L. These data suggest that if mixtures of such compounds were fed to an anaerobic culture, and if the total concentration of phenolics was not too high, the fermentable phenolics should be susceptible to degradation.

There is little information in the literature on the anaerobic degradation of mixtures of phenolics. In general, reports dealing with actual wastewaters, monitor the reduction of phenols as a group or as a loss of COD rather than individual compounds (Dague, 1981; Khan et al. 1982). In a study using pure substrates, Chmielowski and Wasilewski (1966) added p-cresol and resorcinol (each at near 500 mg/L) to a culture adapted to the fermentation of both compounds. Their analyses of the batch culture fluid showed that the resorcinol concentration had essentially dropped to zero, before there was any loss of p-cresol.

This chapter describes two sets of experiments in which batch cultures were subjected to synthetic mixtures of phenolics. In the first experiments, cultures containing both phenol and p-cresol together, and in mixtures with four other alkyl phenolics, were monitored for the loss of substrate and subsequent methane production. In the second set of experiments, m-cresol alone and in combinations with phenol, p-cresol and/or o-cresol was added to batch cultures. Tests with cultures fed industrial wastewaters (described in chapter 9) suggested that m-cresol was being removed from the wastewaters which contained a variety of phenolic compounds. These observations indicated that the presence of other phenolics enhanced the degradation of m-cresol. The preliminary observations were verified using synthetic mixtures of phenolics.

7.1 Procedures

7.1.1 Fermentability of Phenol and p-Cresol in Mixtures of Non-fermentable Phenolics

Table 7.1 summarizes the concentrations of phenolics used in each triplicate set of batch cultures containing 4 mL medium (section 3.1), 1 mL substrate solution and 5 mL domestic anaerobic sludge. For trial 1, three stock solutions were prepared as follows:

Solution 1: o-cresol 3 970 mg/L; p-cresol 4 000 mg/L;
2,6-dimethylphenol 4 230 mg/L;
2,5-dimethylphenol 3 440 mg/L; and
3,5-dimethylphenol 3 610 mg/L.

Solution 2: phenol 10 000 mg/L.

Table 7.1 Concentrations of phenolics in batch cultures receiving mixtures of these compounds.

Trial No.	Culture Label	Phenolic concentrations (mg/L)						Total phenolic concentration (mg/L)
		phenol	o-cresol	p-cresol	2,6-DMP[1]	2,5-DMP	3,5-DMP	
1	Control	0	0	0	0	0	0	0
	A	500	198	200	211	172	180	1 461
	B	250	99	100	106	86	90	731
	C	500	0	0	0	0	0	500
	D	0	0	200	0	0	0	200
	E	500	0	200	0	0	0	700
	F	250	0	100	0	0	0	350
2	Control	0	0	0	0	0	0	0
	1.5B	325	143	133	164	154	147	1 116
	B	250	95	89	109	103	98	744
	0.5B	125	48	44	55	51	49	372

[1] DMP = Dimethylphenol

Solution 3: p-cresol 4 000 mg/L.
With suitable dilutions and combinations, solutions with concentrations 10 times those listed in Table 7.1 were obtained and a single 1 mL aliquot of each was be dispensed into the appropriate culture bottle.

Only one phenolic solution was prepared for trial 2. It contained phenol 3 750 mg/L, o-cresol 1 430 mg/L, p-cresol 1 330 mg/L, 2,6-dimethylphenol 1 640 mg/L, 2,5-dimethylphenol 1 540 mg/L and 3,5-dimethylphenol 1 470 mg/L. Suitable aliquots (1 mL, 0.67 mL and 0.33 mL) of this solution were added to the culture bottles along with appropriate volumes of water (0 mL, 0.33 mL and 0.67 mL, respectively) to give a total volume of 1 mL. Then 4 mL medium were added and each bottle was inoculated with 5 mL sludge.

These cultures were incubated at 37°C with frequent GC analyses to determine methane, phenol and p-cresol concentrations.

7.1.2 Fermentability of m-Cresol Alone and in Mixtures of Phenolics

The various combinations of phenol and the three isomers of cresol used in these batch cultures are summarized in Table 7.2. These solutions were added to 4 mL medium and triplicate sets were inoculated with 5 mL domestic sewage sludge. The final concentration of each phenolic was near 100 mg/L. The cultures were incubated at

Table 7.2 Phenolics added to batch cultures to test the fermentability of m-cresol.

	Volume added (mL)				
Label	Phenol (4 000 mg/L)	o-Cresol (4 000 mg/L)	m-Cresol (4 000 mg/L)	p-Cresol (4 000 mg/L)	Water
Control	0	0	0	0	1
ph	.25	0	0	0	.75
o	0	.25	0	0	.75
m	0	0	.25	0	.75
p	0	0	0	.25	.75
m-ph	.25	0	.25	0	.5
m-o	0	.25	.25	0	.5
m-p	0	0	.25	.25	.5
m-ph-o	.25	.25	.25	0	.25
m-ph-o	.25	0	.25	.25	.25
m-o-p	0	.25	.25	.25	.25
m-ph-o-p	.25	.25	.25	.25	0

37°C and throughout the incubation period, methane and phenolic concentrations were determined. For the substrate analyses, a standard solution containing accurately known concentrations of phenol, o-cresol and m-cresol (each at approximately 100 mg/L) was prepared. Concentrations of phenolics in the cultures were calculated by simple ratio comparisons between the areas of the individual substrate peaks in the sample with those in the standard.

7.2 Results and Discussion

7.2.1 Fermentability of Phenol and p-Cresol in Mixtures of Non-fermentable Phenolics

There were three reasons for choosing the six phenolics used in this study. First, they are all commonly found in phenolic wastewaters from energy related industries, with phenol and the cresols dominant for coal conversion wastewaters. Second, phenol and p-cresol were known to be fermentable while the other four phenolics (Table 7.1) were not, but individually they did not inhibit the anaerobic process at the concentrations used. Third, all six could be resolved using the direct aqueous injection method and the Polyphenyl ether coated Tenax GC column (Bartle et al., 1977).

In trial 1, cultures A and B most closely represent a true wastewater in that they contained the largest variety of compounds (Table 7.1). Cultures C and D were included as positive controls with only the individual fermentable phenolics present in each case. Mixtures of the degradable phenolics at two different concentrations were tested in cultures E and F.

Methane concentrations in the positive control cultures (C) containing phenol at 500 mg/L became higher than those in the phenol-free controls between day 20 and 27. Enhanced methane production occurred between days 12 and 20 for culture D containing 200 mg/L p-cresol. Both of these acclimation times agree with values given in Table 5.4.

Figure 7.1 shows the substrate loss and subsequent methane production observed in culture F which contained nearly 250 mg/L phenol and 100 mg/L p-cresol. By day 13 there was a substantial loss of phenol and by day 19 p-cresol degradation was apparent. Methane concentrations were above those in the controls by day 20. These data indicate that when both of these substrates were present, the culture first became acclimated to phenol and then to p-cresol. However, this preference was not as extreme as was the case with resorcinol and p-cresol (Chmielowski and Wasilewski, 1966) in which the former was completely lost from the culture before the latter began to be degraded. Although phenol is an intermediate in the anaerobic breakdown of p-cresol (Balba _et_ _al_., 1981) it did not appear to accumulate in the medium. For example, on day 25 the average concentration of p-cresol in the three cultures was 58 mg/L indicating a loss of near 42 mg/L. On the same day,

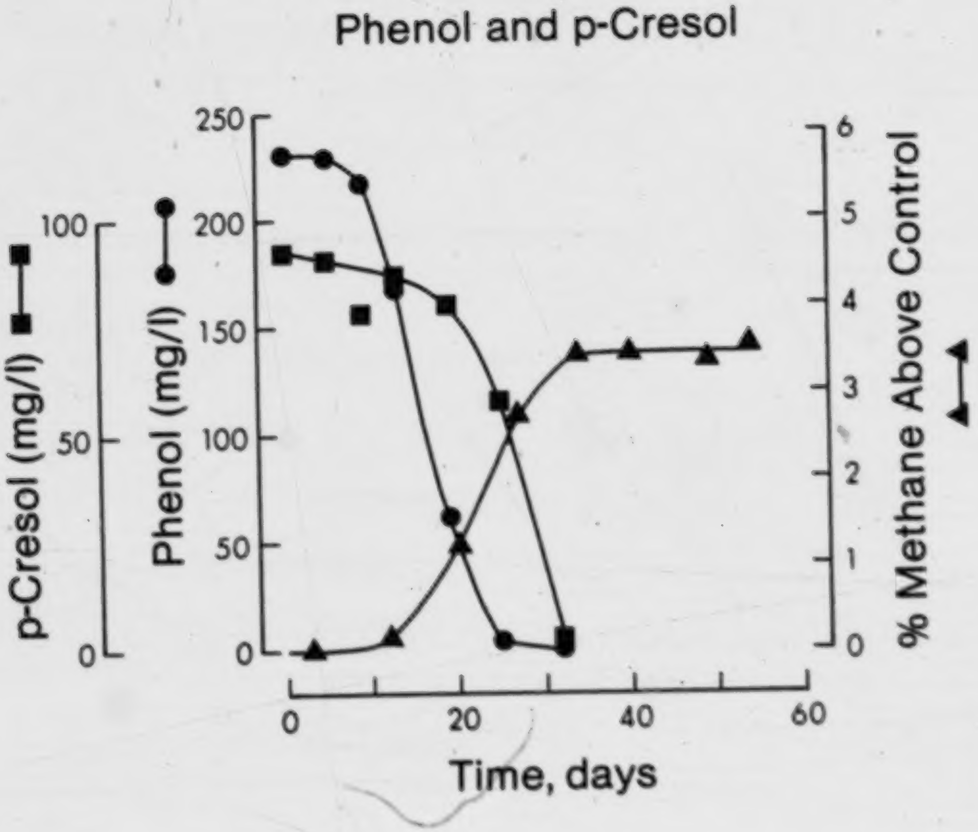


Figure 7.1 Substrate loss and methane production in batch culture F (trial 1) containing phenol and p-cresol.

the average concentration of phenol was only 4 mg/L which suggests that this intermediate is being rapidly metabolized.

Culture E which contained near 500 mg/L phenol and 250 mg/L p-cresol (a total of near 750 mg/L phenolics) showed the same trends as culture F (Figure 7.1). However, all acclimation times were longer, as would be expected with increased phenolic concentrations. These cultures required 38 days and 47 days to cause substantial decreases in the phenol and p-cresol concentrations, respectively. Methane concentrations in excess of the control values occurred between days 54 and 62.

When the six phenolics were present at a total concentration of near 1 461 mg/L (culture A), the activity of the culture was greatly reduced and methane production was immediately inhibited. The culture was unable to degrade phenol or p-cresol. However, in culture B where the concentrations of each phenolic was halved (giving a total near 730 mg/L), phenol and p-cresol were degraded (Figure 7.2). A reduction in the concentrations of both of these substrates was noted between days 25 and 32. During the early stages of incubation, the mean methane concentration in the B cultures fell below that of the control but by day 34 there was significantly more methane in the phenolic-containing culture. Figure 7.3 compares the chromatograms obtained from one of the culture B serum bottles at the time of inoculation with that after 49 days incubation. This clearly shows selective

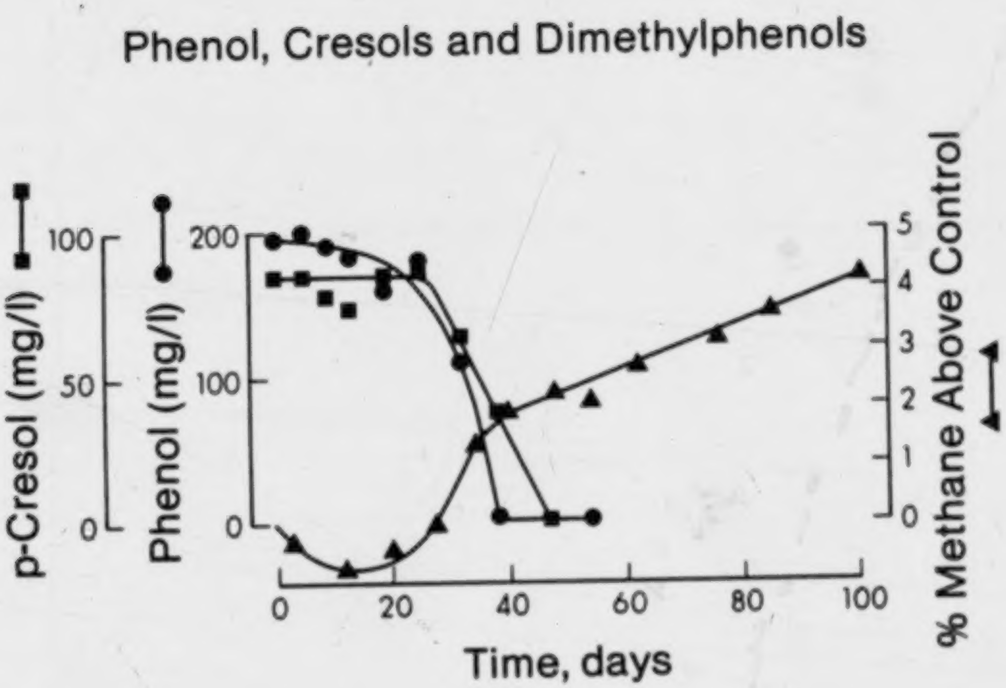


Figure 7.2 Substrate loss and methane production in batch culture B (trial 1) containing a mixture of six phenolics with a total concentration near 730 mg/L.

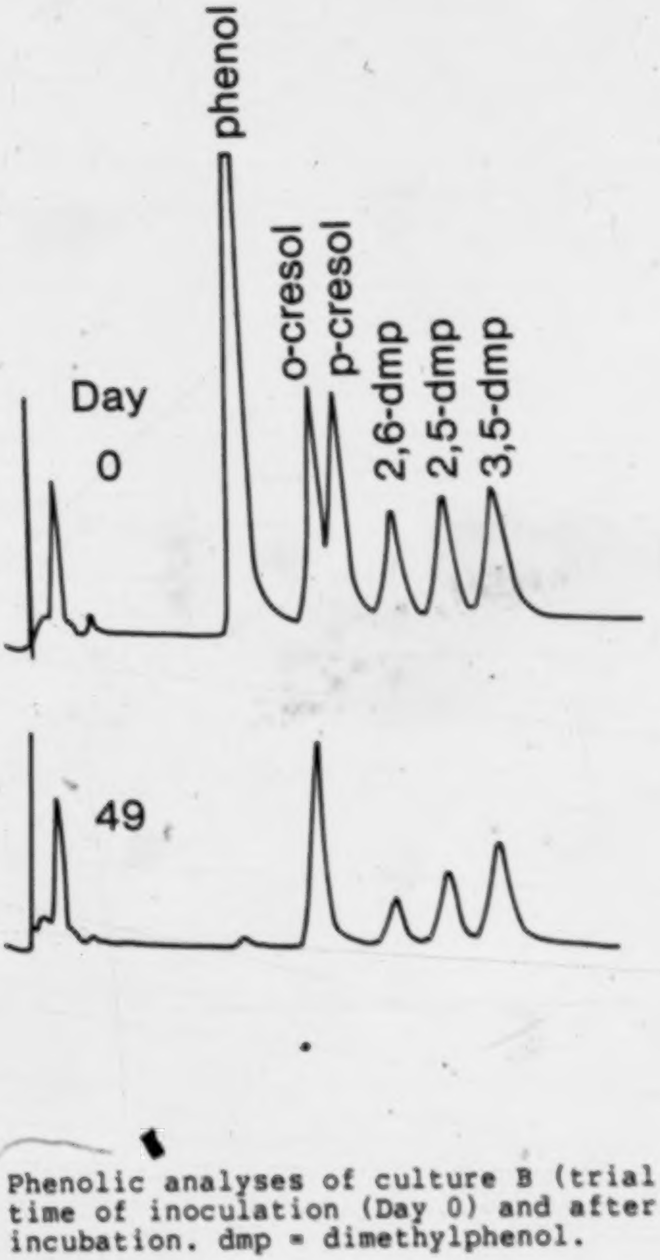


Figure 7.3 Phenolic analyses of culture B (trial 1) at the time of inoculation (Day 0) and after 49 days incubation. dmp = dimethylphenol.

removal of phenol and p-cresol from this mixture of six phenols.

The experiments in the trial 1 using a mixture of six phenolics showed that when the total concentration was near 730 mg/L (culture B) degradation of phenol and p-cresol occurred. However no degradation occurred at double this concentration (culture A). In trial 2, a different range of concentrations of the six phenolics was tested. Culture B in this trial had nearly the same individual and total phenolic concentrations as did culture B in trial 1. Cultures containing one-half (designated 0.5B) and three-halves of these concentrations (designated 1.5B) were established. Of these three, only culture 0.5B, which contained a total of near 372 mg/L phenolics (Table 7.1) actively degraded phenol and p-cresol. The substrate loss and methane production are shown in Figure 7.4. These results are similar to those in Figures 7.1 and 7.2 except in this case the removal of p-cresol does not appear to commence until virtually all of the phenol has been lost.

In the early screening experiments with individual compounds (sections 5.2.1 and 5.2.4), some sludge samples used for batch cultures were found to degrade p-cresol at 400 mg/L while other samples would not. The conflicting results from the B cultures in trials 1 and 2, inoculated with municipal sludges taken on different days, appear to present a situation which is analogous to the p-cresol case. That is, at phenolic concentrations near inhibitory levels,

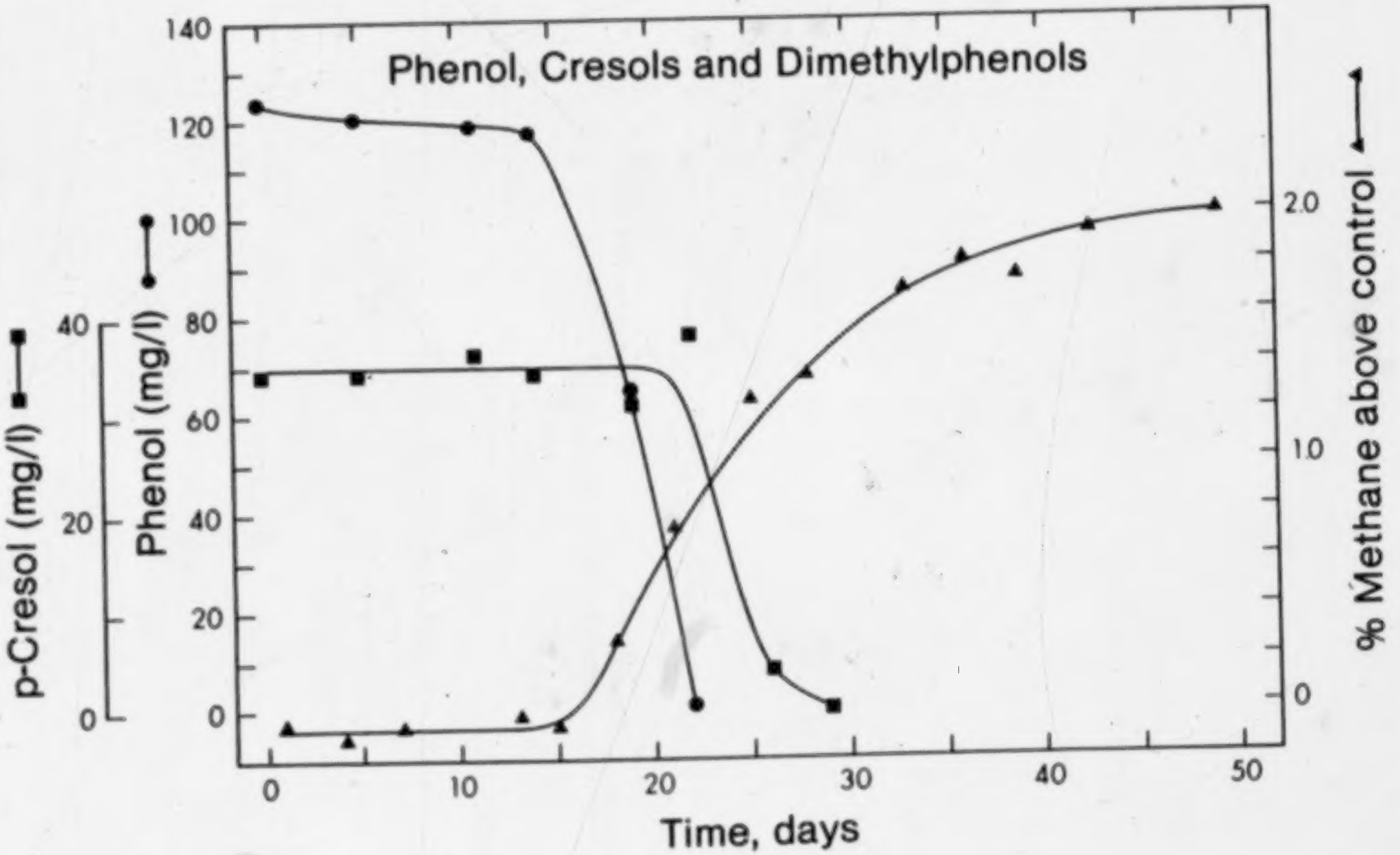


Figure 7.4 Substrate loss and methane production in batch culture 0.58 (trial 2) containing a mixture of six phenolics with a total concentration near 372 mg/L.

the ability to establish active cultures was quite variable.

7.2.2 Fermentability of m-Cresol Alone and in Mixtures of Phenolics

At the time these batch cultures were inoculated, there had been no reports in the literature demonstrating m-cresol degradation by anaerobic consortia yielding methane. However in July 1983, prior to the end of the incubation period, Boyd _et_ _al_. (1983) reported that m-cresol could be degraded by organisms in domestic anaerobic sludge. In their cultures, the methane concentration was observed to be in excess of that in the control after four weeks and the m-cresol concentration had dropped from 50 mg/L to an undetectable level after seven weeks.

Healy and Young (1979) reported that certain cultures which had been enriched on one phenolic compound were able to almost immediately begin to ferment another compound with similar chemical structure. For example, a culture acclimated to vanillic acid metabolism was "cross-acclimated" (or co-enriched) to six other compounds including vanillin, catechol and syringic acid. The experiment described here, attempted to establish active phenol and p-cresol degrading cultures which would be cross-acclimated to the fermentation of m-cresol that was included in the medium.

Figure 7.5 shows the substrate loss and methane production from cultures which contained one of the following as their sole phenolic substrate: phenol, p-cresol or m-cresol.

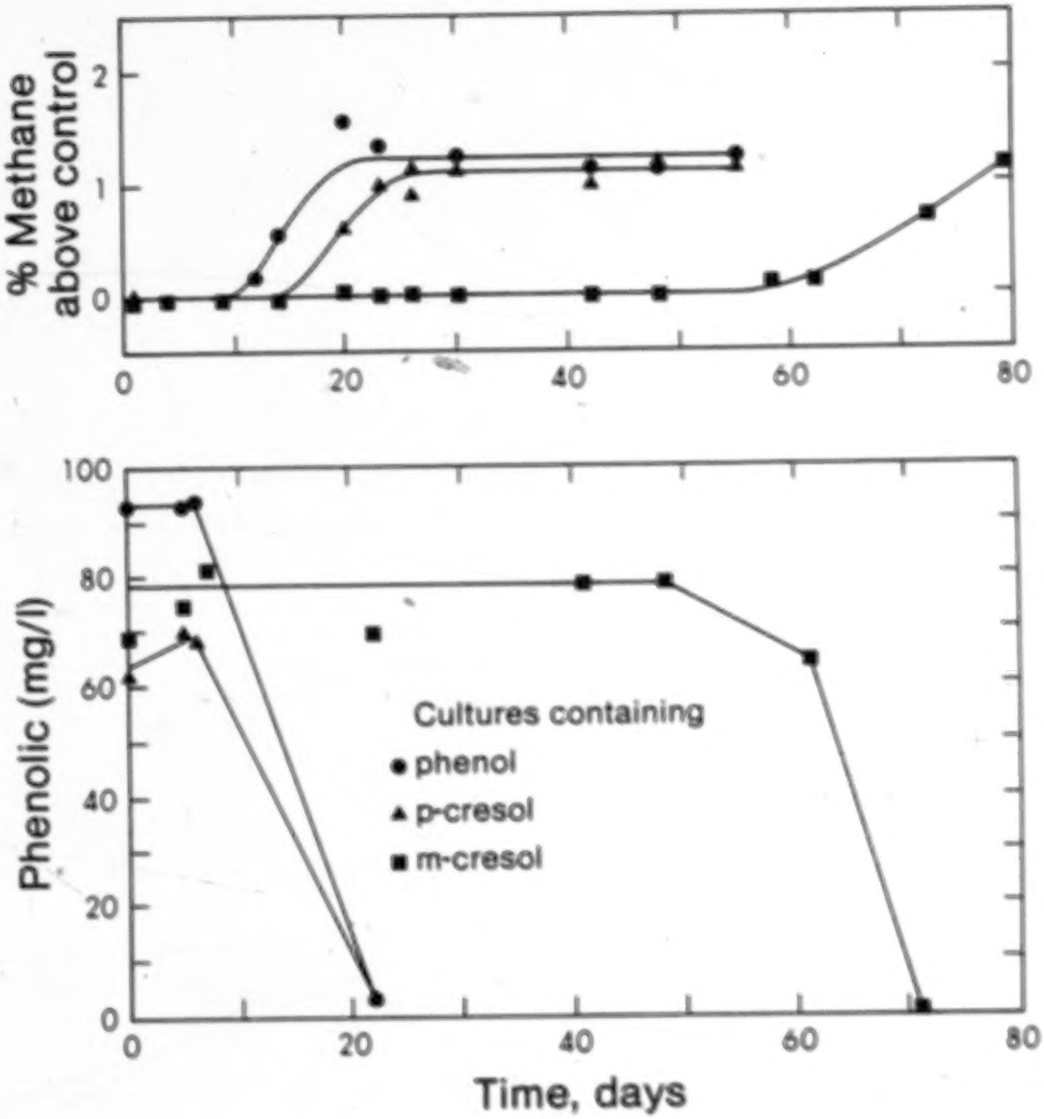


Figure 7.5 Substrate loss and methane production in batch cultures receiving individual phenolics.

As had been observed earlier, phenol and p-cresol were readily degraded and by days 14 and 20, respectively, enhanced methane production was observed. By day 22, the concentrations of these substrates were near zero. When m-cresol was the only phenolic present, its loss from the cultures was noted on day 61 and by day 71 it was no longer detectable. Methane production from this substrate was apparent by day 71.

Results presented earlier in this report suggested that m-cresol was not degradable under anaerobic conditions. These experiments differed from the early experiments in two respects. First the incubation times for the previous cultures never exceeded 45 days. Figure 7.5 shows that an acclimation time of about 60 days was required by these batch cultures. The production of methane from this substrate and the complete loss of m-cresol were observed after 61 and 71 days, respectively. These were considerably longer than the corresponding times of four and seven weeks reported by Boyd et al. (1983). Second, the lowest initial concentration used in the early experiments was 200 mg/L (Table 5.2) whereas the initial concentration in this series of batch cultures was approximately 80 mg/L (Figure 7.5). The higher initial concentrations may have been inhibitory to the m-cresol degraders.

The inability to resolve the m- and p- isomers of cresol by the GC method used, complicates the interpretation of the data obtained from cultures containing a mixture of these substrates. Here, a single concentration has been reported from the sum of the concentrations of these two isomers and it has been designated as m/p-cresol. The data in Figure 7.6 show the concentrations of phenol and m/p-cresol from two sets of cultures which contained all three compounds. One set also contained o-cresol. In both cases, phenol was the first to be removed from the culture medium. Between day 22 (when the phenol concentrations were essentially zero) and day 28 there was an extreme drop in the m/p-cresol concentrations. This decrease was very likely the result of p-cresol degradation and this pattern of substrate loss was consistent with those observed in Figures 7.1, 7.2 and 7.4 from cultures which contained mixtures of phenolics devoid of m-cresol. In both of the cultures shown in Figure 7.6, there was a seven to eight day period over which the m/p-cresol concentrations remained near 75 mg/L. Over this period there was likely no p-cresol present, and the cultures were presumably acclimating to m-cresol. This would agree with data in Figure 7.5 which clearly show that m-cresol degradation lags far behind that of p-cresol. m-Cresol was removed from both cultures by day 71.

The presence of other fermentable phenolics in batch cultures shortened the acclimation time for m-cresol degradation as shown in Figure 7.7. In each culture containing m-cresol, the first sampling day on which its mean concentration was measured as 60 to 70 mg/L was noted. These times indicated that m-cresol degradation had started

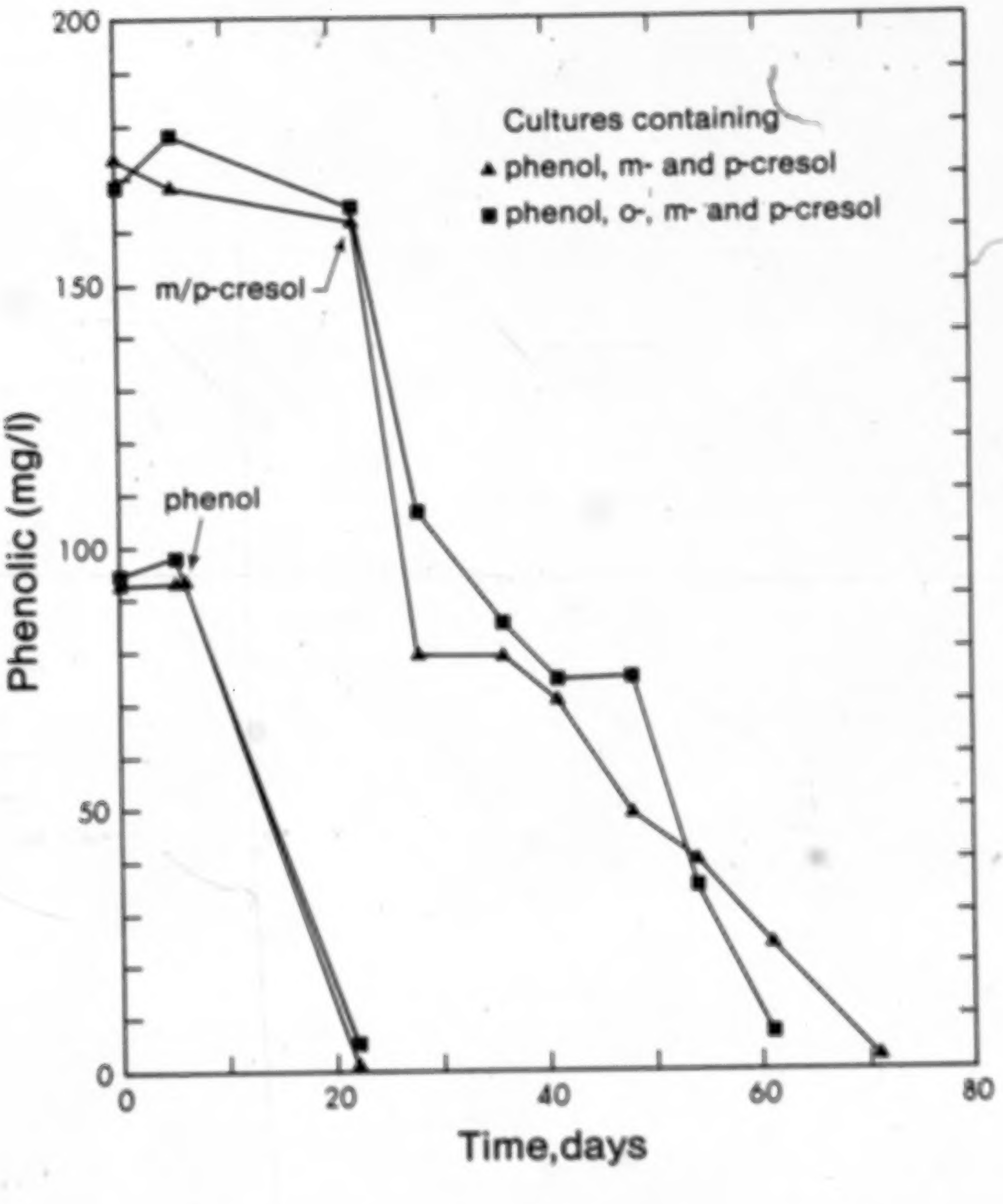


Figure 7.6 Substrate loss from batch cultures receiving m-cresol mixed with other phenolics.

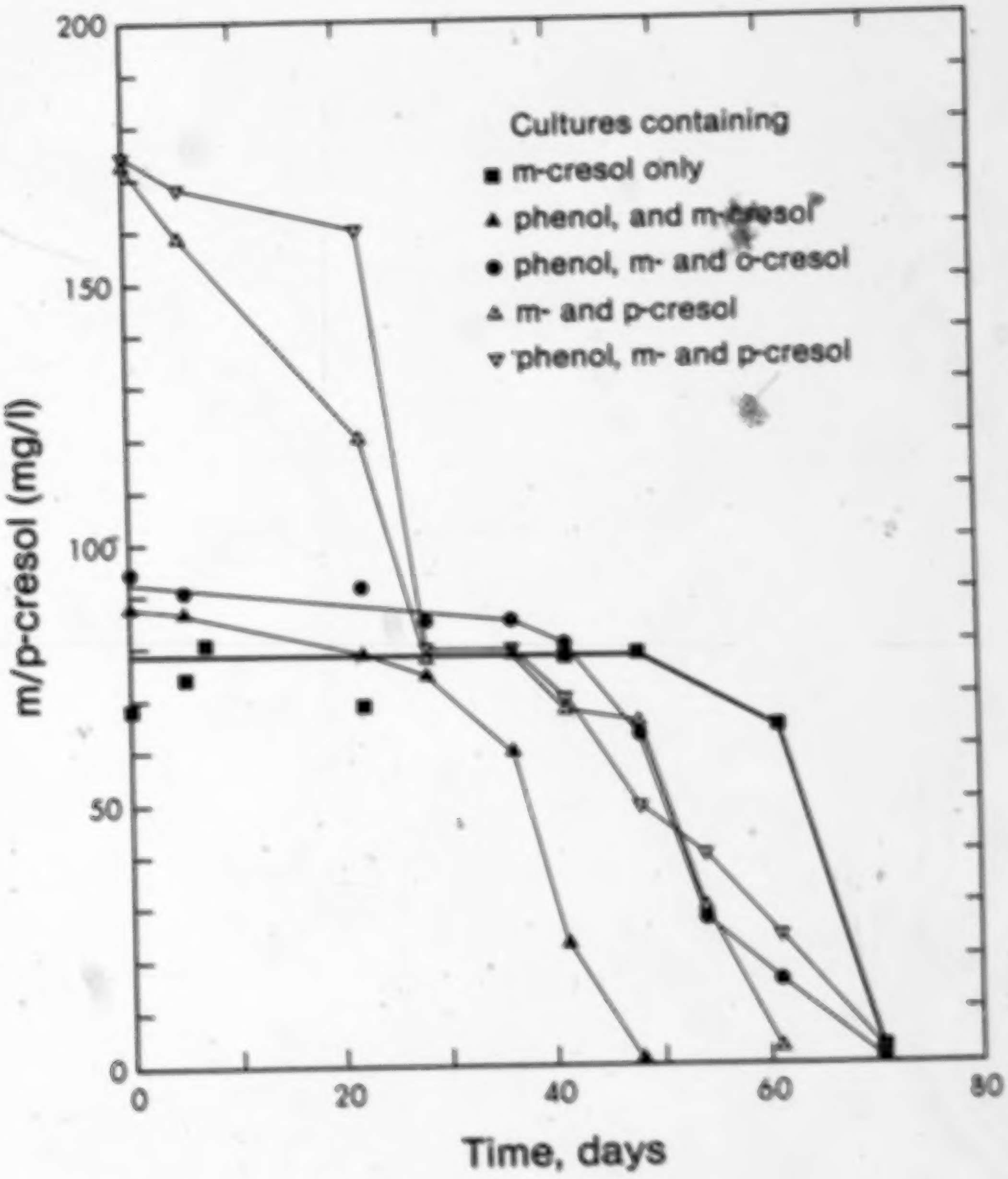


Figure 7.7 Reduction of the acclimation time for m-cresol degradation when other phenolics were present in batch cultures.

and they were used to determine to what extent the presence of other fermentable phenolics had decreased the acclimation time for m-cresol.

Batch cultures containing only m-cresol required 61 days to acclimate to this substrate. However, with phenol present at 100 mg/L, the m-cresol concentration reached 60 mg/L after 36 days (Figure 7.7). Thus there was a 25-day reduction in the m-cresol acclimation time when the cultures were previously adapted to phenol degradation. The mean m-cresol concentration in the cultures containing phenol, o- and m-cresol reached 65 mg/L on day 48. This mixture shortened the acclimation time for m-cresol by 13 days.

The presence of approximately 80 mg/L of the non-fermentable compound, o-cresol along with phenol lengthened the acclimation time by 12 days over that observed with only phenol and m-cresol in batch cultures. A similar trend was observed in Figure 7.6 where the cultures without o-cresol began to degrade m-cresol by day 41 while those with o-cresol required over 48 days to commence m-cresol degradation.

The cultures which contained a mixture of m- and p-cresol (Figure 7.7) showed a loss of m-cresol on day 48 which represents a 13 day decrease in the m-cresol acclimation time. When phenol, m- and p-cresol were present, the acclimation time for m-cresol degradation was about 20 days less than when m-cresol alone was in the cultures. Thus, in all four cases shown in Figure 7.7, mixtures of fermentable phenolics and m-cresol reduced the acclimation time for m-cresol degradation in batch cultures.

Methane production in excess of the controls was observed in all of the 10 cultures (Table 7.2) containing fermentable phenolics. Cultures with more than one fermentable test compound always showed distinct, stepwise increases in methane as illustrated by two examples in Figure 7.8. In the first example, the culture was fed a binary mixture containing phenol and m-cresol. Between days 12 and 23, there was a sharp increase in methane concentration corresponding to the interval during which phenol was lost from the culture (data not shown). The methane concentration then remained constant between days 23 and 30 after which there was another increase in the methane level corresponding to the degradation of m-cresol (Figure 7.7). The second example in Figure 7.8 shows the methane produced in the cultures which received a mixture of phenol, m- and p-cresol. Between days 14 and 30 there was a burst of methane production. During this time, the concentrations of phenol and, presumably, p-cresol dropped extensively in these cultures (Figure 7.6). Over the period between days 30 and 42, there was no increase in methane concentration (Figure 7.8) and only a slight decrease in the m-cresol concentration (Figure 7.6). As m-cresol was degraded in these cultures, a corresponding increase in methane was observed between days 48 and 79.

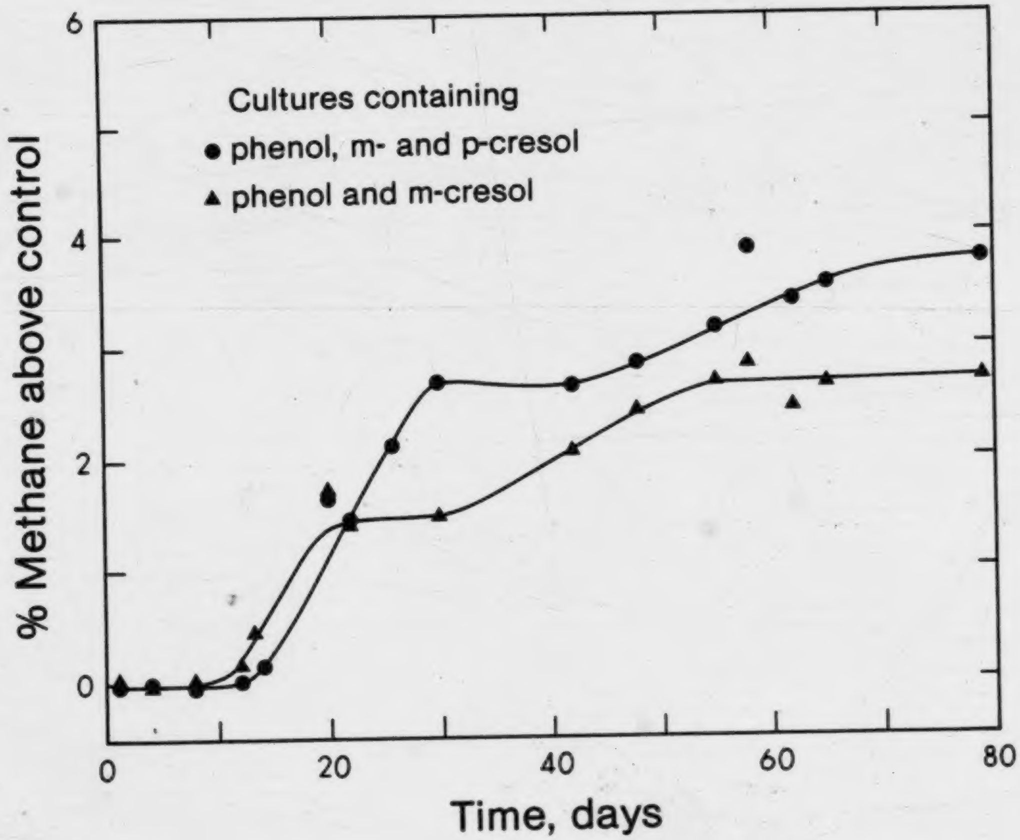


Figure 7.8 Typical methane production curves from batch cultures receiving m-cresol mixed with other fermentable phenolics.

The results obtained from the investigation of m-cresol fermentation in the presence of other degradable phenolics indicate that possibly a wider range of phenolics - which were previously thought to be nondegradable under anaerobic conditions - may be attacked by cultures that have been previously enriched on other similar compounds. However, no other mixtures of fermentable and "nondegradable" phenolics were tested during this study.

The microbiology of the phenolic degrading mixed population is not yet understood. The initial attack on the three phenolics - phenol, p-cresol and m-cresol - may be due to three different organisms, each exhibiting a different acclimation time. However, since the presence of a readily fermentable phenolic (phenol and/or p-cresol) shortened the acclimation time for m-cresol degradation, it is possible that the same organism which attacks the former compounds is also responsible for the degradation of m-cresol. Prior growth at the expense of the more easily degradable phenolics would selectively increase the numbers of phenolic degraders which may be the reason for the reduced acclimation times observed for m-cresol when other fermentable phenolics were present.

8. BATCH CULTURE FERMENTATIONS OF PHENOLIC WASTEWATERS

Batch culture experiments described in the previous chapter showed that fermentable phenolics could be selectively removed from synthetic mixtures containing several phenolics. These results indicated that their removal from industrial wastewaters might be possible. Dearborn Environmental Consulting Services supplied two phenolic wastewaters for this study. One was a combination of blast furnace blowdown (30%) and ammonia-stripped waste ammonia liquor from coal coking pyrolysis gas quenching drums (70%) from a steel plant in Hamilton, Ontario. The other was a coal conversion wastewater from a pilot scale H-coal process in Catlettsburg, Kentucky. Throughout the following text these are referred to as the coke effluent and the H-coal effluent, respectively. The phenolic content of the former was 410 mg/L and the latter was 7 600 mg/L (Dearborn, personal communication).

Various dilutions of each of these wastewaters were added to unsupplemented and VOA-supplemented batch cultures to determine whether the fermentable phenolics could be removed and whether the wastewater exerted any inhibitory effects. These wastewaters differed from the synthetic phenolic mixtures in that a variety of unidentified and unquantitated non-phenolic organic compounds and many inorganic ions were also present.

When the concentration of wastewater in a batch culture became inhibitory to methanogenesis, the cause was not necessarily the phenolic concentration as was the case with the synthetic mixture used in the earlier parts of this study. The inhibition may have been due to the inorganic or non-phenolic organic components in the wastewater. To establish whether the organic or inorganic components were more inhibitory, the wastewaters were exhaustively extracted with diethyl ether and the aqueous phase (containing mainly inorganic ions) was added at various concentrations to batch cultures containing phenol as a fermentable substrate. Inhibition of substrate degradation and/or methane production in these cultures would indicate that the inorganic and/or nonextractable portion of the wastewater contained toxic materials.

Because of its very high phenolic concentration, further emphasis was directed toward assessing the treatability of the H-coal effluent. This wastewater was, therefore, used in two additional batch culture studies. One involved the preparation of a "reconstituted" H-coal effluent which was done by exhaustively extracting the organics from the original wastewater and then adding the reagent grade phenolics back to the extracted aqueous phase. This reconstituted H-coal effluent had the same phenolic concentrations as the original wastewater but was devoid of the other ether-extractable organics. The fermentability and inhibitory effects of the reconstituted H-coal was then tested in batch cultures.

The second additional study with H-coal effluent involved the determination of the ultimate amount of methane produced per unit volume of wastewater. These data were to be used to evaluate the performance of semicontinuous cultures described in chapter 9.

8.1 Procedures

8.1.1 Analyses of the Two Industrial Wastewaters

8.1.1.1 Major Phenolic Compounds

The coke effluent was analyzed undiluted while two dilutions of H-coal effluent were prepared in distilled water. One solution was a 1 in 25 dilution while the other was a 1 in 5 dilution. Each of these was analyzed by injecting 3 μL aliquots into the Polyphenyl ether coated Tenax GC column. Retention times of eluting peaks were matched with those of authentic standards and peak areas were compared to those of standards to determine the concentrations of each component. For the H-coal effluent, phenol, o-cresol and m/p-cresol concentrations were determined from the more dilute solution (1 in 25) while the concentrations of the dimethylphenols were determined from the more concentrated solution (1 in 5).

To determine whether both the m- and p-isomers of cresol were present and, if they were, to find the relative proportion of each, another GC method capable of resolving these isomers was used. This method used a 3 m x 2 mm ID glass column packed with GP 80/100 mesh Carbopack C/0.1% SP-1000 (Supelco, Inc.) in a Hewlett-Packard (model 5736) GC with nitrogen as carrier gas at 13 mL/min. The oven temperature was 225°C while the injection port and detector were at 250°C. Air and hydrogen flowed into the flame ionization detector at 240 and 30 mL/min respectively.

Since aqueous injections could not be used with this GC method, a 250 mL aliquot of each wastewater was acidified to pH 1.5 and extracted with methylene chloride according to the procedure of Stuermer *et al*. (1982). The methylene chloride was back-extracted with 0.01 M KOH leaving the neutral compounds in the organic solvent. The aqueous phase was adjusted to pH 1.5 and extracted with methylene chloride to recover the acidic compounds including the phenolics. After drying over Na_2SO_4, a 1 μL aliquot was injected into the GC. Retention times of peaks in the extract were matched to those of authentic standards.

8.1.1.2 Volatile Organic Acids

VOAs were analyzed using a MicroTek model MT220 GC equipped with a flame ionization detector. A 1.8 m x 2 mm ID glass column packed with GP 10% SP-1000/1% H_3PO_4 on Chromosorb W AW (100/120) was kept at 130°C with nitrogen carrier gas flowing at 30 mL/min. The injection port and detector temperatures were 155° and 200°C, respectively. Peak areas were recorded with a Hewlett-Packard 3390A integrator.

Prior to injection, nine parts of a sample or standard mixture were combined with one part 3 M H_3PO_4. A volume of 0.5 μL was injected. Some samples were concentrated prior to analysis. In these cases, the entire 10-mL culture was placed in a beaker and the pH adjusted to approximately 10 with NaOH. This was dried at 80°C overnight and then resuspended in 1 mL 0.3 M H_3PO_4. A 0.5 μL aliquot of this 10 fold concentrate was then analyzed.

8.1.2 Batch Cultures Receiving Unextracted Wastewaters

8.1.2.1 Coke Effluent

In order to minimize the chance of altering the composition of the coke effluent during autoclaving, this wastewater was prereduced and added to the sterile, prereduced medium just prior to inoculation. An 80 mL aliquot of the wastewater was placed in a 158 mL serum bottle and the pH adjusted to near 7 with 1 M HCl. After the addition of 0.8 mL resazurin solution, the bottle was sealed and the headspace gas exchanged 10 times to remove O_2. Each exchange was done by first evacuating the headspace and then filling it with O_2-free 30% CO_2 in N_2. After this procedure was complete, 0.8 mL sodium sulfide solution was added.

The final concentrations of coke effluent in the 10 mL culture were 10, 20, 25, 30, 40, and 50% (V/V). These concentrations were achieved by injecting volumes of 1, 2, 2.5, 3, 4, and 5 mL coke effluent into a series of bottles containing prereduced aliquots of 2 mL concentrated medium (equation 3.1) and respectively 4, 3, 2.5, 2, 1 and 0 mL water. Inoculation with 3 mL domestic sludge into each bottle gave a total culture volume of 10 mL. Controls containing 5 mL of water in place of the coke effluent were also prepared.

Another series of cultures was set up in exactly the same manner and each bottle was supplemented with 0.1 mL VOA solution (37.5 mg/mL acetic acid and 13 mg/mL propionic acid, pH 7).

8.1.2.2 H-coal Effluent

An aliquot of the H-coal effluent was prereduced in the same manner as the coke effluent. However no pH adjustment was required. The final concentrations of H-coal effluent in the batch cultures were 2, 4, 6, 8, 10, 12 and 15% (V/V). Aliquots (0.2 to 1.5 mL) of prereduced H-coal effluent were injected into culture bottles containing 3 mL prereduced concentrated medium and appropriate volumes of water (1.8 mL to 0.5 mL, respectively). Five mL domestic anaerobic sludge were added to each bottle to give a total volume of 10 mL. Cultures were established on both VOA-supplemented and unsupplemented medium. Control cultures contained no H-coal effluent.

All cultures were incubated at 37°C with frequent methane and substrate analyses.

8.1.3 Batch Cultures Receiving Ether-extracted Wastewaters

8.1.3.1 Extracted Coke Effluent

After adjusting the pH to 7, a 200 mL aliquot of coke effluent was extracted seven times with 30 mL portions of diethyl ether. The aqueous phase was then drained into a beaker and left overnight to allow the dissolved ether to evaporate. The extent of the phenolic removal and the presence of any residual ether were determined by GC analysis. A portion of this extracted coke effluent was prereduced and cultures containing 10, 20, 30, 40, and 50% (V/V) were established in the same manner as done with the unextracted coke effluent. Both VOA-supplemented and unsupplemented media were used. To provide the culture with a fermentable phenolic substrate, 200 mg/L phenol were included in the medium.

8.1.3.2 Extracted H-coal Effluent

Two hundred mL of H-coal effluent were ether extracted and analyzed as outlined above. Two concentration ranges of the extracted H-coal were tested in separate batch culture experiments. In the first experiment, 0.2, 0.8, 1.0, 1.5 and 2.5 mL extracted H-coal effluent were added to bottles containing 1.5 mL concentrated medium, 1 mL phenol solution (2 000 mg/L) and appropiate aliquots of water to give a volume of 5 mL after the addition of the extracted H-coal effluent. Each bottle was inoculated with 5 mL domestic anaerobic sludge giving final extracted H-coal concentrations of 2, 8, 10, 15 and 25% (V/V).

In the second experiment, the cultures received 2.5, 3, 3.5, 4, 4.5 and 5.0 mL aliquots of prereduced extracted H-coal effluent. These volumes were injected into bottles containing 1 mL concentrated medium, 1 mL phenol solution (2 000 mg/L) and sufficient amounts of distilled water to give a volume of 7 mL after the addition of the aliquots of extracted H-coal effluent. After inoculating with 3 mL domestic anaerobic sludge, the final concentrations of extracted H-coal effluent were 25, 30, 35, 40, 45 and 50% (V/V).

For each experiment using the ether-extracted wastewaters, two types of control cultures were established. One contained phenol (200 mg/L final concentration) but no extracted wastewater. The other control contained no phenol and no extracted wastewater. Distilled water was added in place of these solutions to provide a final culture volume of 10 mL. All cultures were incubated at 37°C with frequent methane and phenol analyses.

8.1.4 Cultures Receiving Reconstituted H-coal Effluent

8.1.4.1 Preparation of Reconstituted H-coal Effluent

Based on the results of the analyses of H-coal effluent, seven concentrated phenolic solutions were prepared in ether-extracted H-coal effluent. The volumes and concentrations of each solution combined in a 158 mL serum bottle were as follows: 40 mL phenol (12 250 mg/L), 5 mL o-cresol (9 080 mg/L) 10 mL m-cresol (12 500 mg/L), 5 mL p-cresol (8 030 mg/L), 5 mL 2,5-dimethylphenol (1 260 mg/L), 10 mL 3,5-dimethylphenol (2 130 mg/L) and 5 mL 3,4-dimethylphenol (880 mg/L). A further 18 mL of extracted H-coal effluent and 1 mL resazurin solution were added giving a total volume of 99 mL liquid in the serum bottle which was then sealed. After the headspace gas had been repeatedly evacuated and filled with O_2-free 30% CO_2 in N_2, 1 mL sodium sulfide solution was added to further reduce the redox potential of the reconstituted H-coal effluent.

8.1.4.2 Batch Cultures with Reconstituted H-coal Effluent

Aliquots of 0.2, 0.4, 0.6, 0.8, 0.9, 1.0, 1.2, 1.4 and 1.6 mL prereduced reconstituted H-coal effluent were injected into serum bottles containing 3 mL prereduced concentrated medium with sufficient volumes of distilled water to give a volume of 5 mL in each bottle after the reconstituted H-coal effluent was added. Each bottle was inoculated with 5 mL domestic anaerobic sludge resulting in the final test concentrations of reconstituted H-coal effluent: 2, 4, 6, 8, 9, 10, 12, 14, and 16% (V/V). A control culture containing no reconstituted H-coal effluent was included along with two positive controls which contained 2% and 4% unextracted H-coal effluent respectively. All cultures were incubated at 37°C with frequent methane and substrate analyses.

8.1.5 Ultimate Methane Production from H-coal Effluent

Using a volumetric pipette, 1.0 mL aliquots of H-coal were added to a 158 mL serum bottle containing 4 mL distilled water and 0.5 mL resazurin solution. After gassing the headspace for approximately 3 min with O_2-free 30% CO_2 in N_2, 45 mL domestic anaerobic sludge were added and the bottles were sealed. Control cultures were prepared in the same manner with 1 mL distilled water replacing the H-coal effluent. After the inoculated cultures had been at room temperature for approximately 1 h, the headspace gas pressure was adjusted to atmospheric pressure. These cultures were monitored for methane production as outlined in section 5.1.5.

8.2 Results and Discussion

8.2.1 Chemical Characteristics of the Industrial Wastewaters

Dearborn Environmental Consulting Services provided the analyses of the two industrial wastewaters summarized in Table 8.1. The coke effluent contained phenolics at a concentration of 410 mg/L as determined by the 4-aminoantipyrine method (APHA, 1980). This colourimetric method provides a measure of the combined concentrations of a variety of phenolics including phenol and o- and m-substituted phenolics. The pH of the coke effluent was 11.8 and therefore required adjusting to near neutrality prior to its addition to batch cultures. Of the non-phenolic

Table 8.1 Analyses of phenolic wastewaters received from Dearborn Environmental Consulting Services.(1)

	Coke effluent	H-coal effluent
Organic carbon (mg/L)	524	7 600
COD (mg/L)	5 500	21 100
Phenolics(2)(mg/L)	410	7 600
Total Kjeldahl nitrogen (mg/L)	102	267
Nitrite nitrogen (mg/L)	0.1	0.2
Nitrate nitrogen (mg/L)	0	0.8
Ammonia nitrogen (mg/L)	210	6.4
Total cyanide (mg/L)	8.32	0.21
Total phosphorus (mg/L)	1	5
pH	11.8	7.4

(1) Results provided by Dearborn Environmental Consulting Services

(2) Colourimetric method, equivalent to method 510A (APHA, 1980)

parameters reported, only the cyanide concentration (8.32 mg/L) was high enough to produce toxic effects in the cultures. Yang et al. (1980) and Lettinga et al. (1981) reported that cyanide concentrations of 1 mg/L or more inhibit methane production. The high concentration of phenolics (7 600 mg/L) in the H-coal effluent would cause inhibition in batch cultures.

The data in Table 8.2 summarize the GC analyses of phenol and the alkyl phenolics in the two wastewaters. The sum of alkyl phenolics measured by GC (Table 8.2) agreed reasonably well with the total phenolics measured by the colourimetric method (Table 8.1). The sum by GC accounted for 93% of total phenolics for the coking effluent and 98% for the H-coal effluent. In both wastewaters, phenol was the predominant compound. Considering data in both Tables 8.1 and 8.2, phenol accounted for 78% of the phenolics in the coke effluent and 62% of the phenolics in the H-coal effluent. Since earlier results showed that m- and p-cresol were also fermentable, the complete anaerobic biodegradation of the three compounds would remove 91% of the phenolics from the coke effluent and 86% of the phenolics from the H-coal effluent. Prior experience with pure substrates suggested that neither of these full strength wastewaters would be amenable to anaerobic degradation. Therefore a series of dilutions were tested to determine which concentrations would be fermentable and which would be inhibitory.

Table 8.2. Concentrations of various phenolics in the two industrial wastewaters used in batch culture studies.(1)

	Concentration (mg/L)	
Phenolic	Coke effluent	H-coal effluent
phenol	320	4 900
o-cresol	6	586
m/p-cresol(2)	55(3)	1 650(4)
2,4/2,5-dimethylphenol(2)	ND(5)	63
3,5-dimethylphenol	ND	213
3,4-dimethylphenol	ND	44
Total phenolics	381	7 456

(1) Direct aqueous injection into Polyphenol ether coated Tenax GC column.

(2) Not resolved by this GC method.

(3) Analysis with Carbopack C column showed the m/p ratio to be 6.34. Therefore 47.5 mg/L m-cresol and 7.5 mg/L p-cresol.

(4) Analysis with Carbopack C column showed the m/p ratio to be 2.89. Therefore 1 230 mg/L m-cresol and 420 mg/L p-cresol.

(5) Not detected.

8.2.2 Batch Cultures Receiving Unextracted Wastewaters

8.2.2.1 Coke Effluent

The dilutions of the coke effluent tested ranged from 10% to 50% (V/V) and the methane production by these batch cultures is shown in Figure 8.1. None of the concentrations tested produced methane in excess of the concentrations found in the control cultures. Methane in the cultures containing 10% coke effluent (data not shown in Figure 8.1) was not significantly different from that in the controls. However, increasing wastewater concentrations decreased the rate of methane production and those cultures which contained 40% and 50% (V/V) coke effluent produced small amounts of methane only during the first days of incubation.

Analyses of the phenolics in the culture fluid at the end of the incubation showed that phenol had been completely removed from the cultures which received ≤30% coke effluent. There was no loss from those containing 40% or 50% of this wastewater. No significant loss of m/p-cresol was observed in any of the cultures. When present at 10%, the phenol concentration was initially only 32 mg/L (i.e. 0.32 mg in the 10 mL culture) and the amount of the methane expected (approximately 0.24 mL in 60 mL headspace) would not likely be detected in excess of the control. However, detectable amounts of methane should have been found in those cultures containing 20% to 30% wastewater, but they were not.

This experiment was repeated with the inclusion of a series of triplicate 25% (V/V) coke effluent cultures.

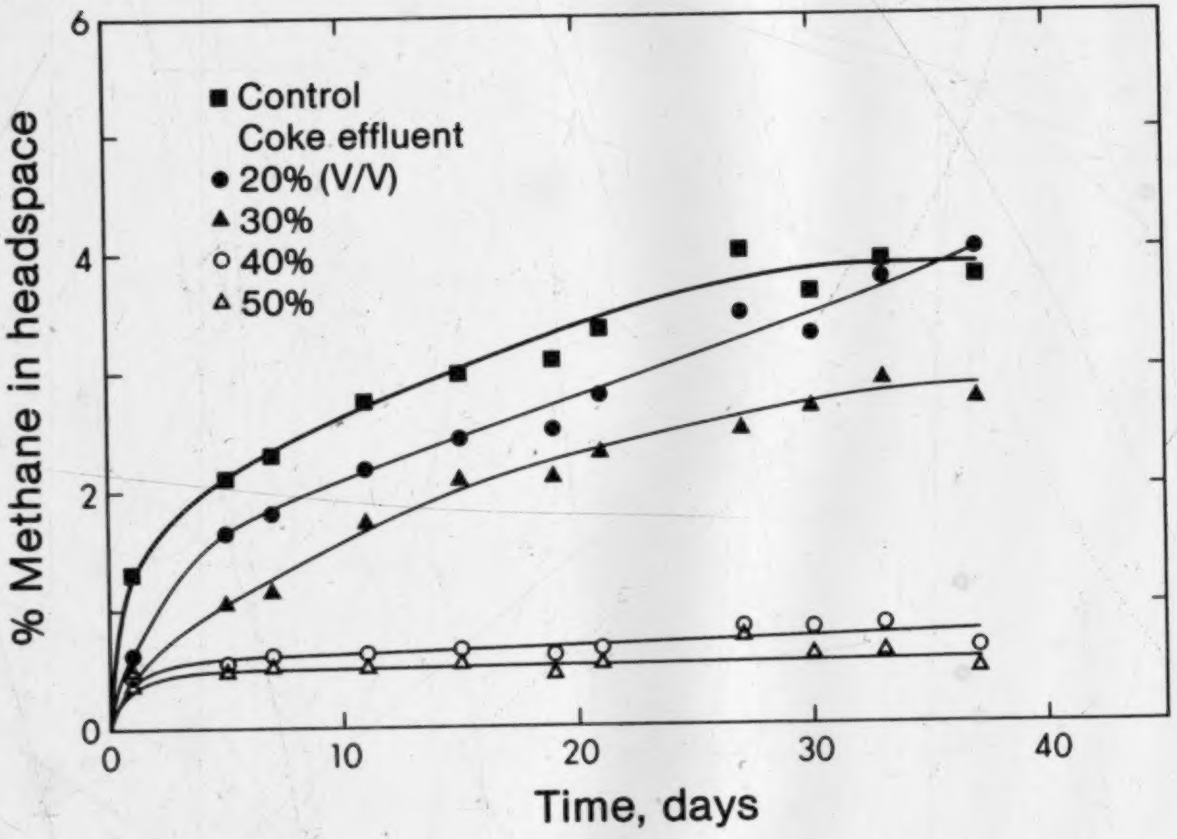


Figure 8.1 Methane production in batch cultures containing various concentrations of coke effluent - without VOA supplementation.

Analyses of the culture supernatants showed that phenol was removed from all those containing ≤30% coke effluent. Again there were no enhanced methane concentrations in agreement with the data in Figure 8.1.

At the end of the 96 day incubation period, the entire volume of the control, 25% and 30% cultures were concentrated and analyzed for VOAs. Only trace amounts of acetic acid were found in all of these cultures. Those which contained the coke effluent had no other VOAs present. These data suggest that the coke effluent at concentrations ≤30% (V/V) does not inhibit the initial attack on the phenol molecule but its subsequent conversion to VOAs and methane does not occur. The fate of the phenol carbon was not determined.

When VOAs were supplemented to cultures containing coke effluent, methane production was inhibited by all five concentrations tested (Figure 8.2). When the coke effluent was present at 10%, methane evolution after one day incubation was only 65% of that in the controls. The extent of inhibition increased as the concentration of coke effluent in the batch cultures increased until there was a 92% reduction in methane production after one day in the cultures containing 50% effluent. Over the 39 day incubation period, mean methane concentrations in the cultures containing 10% to 30% wastewater reached that of the control cultures. Methane concentrations in the 40% and 50% effluent cultures slowly increased but did not reach the levels found

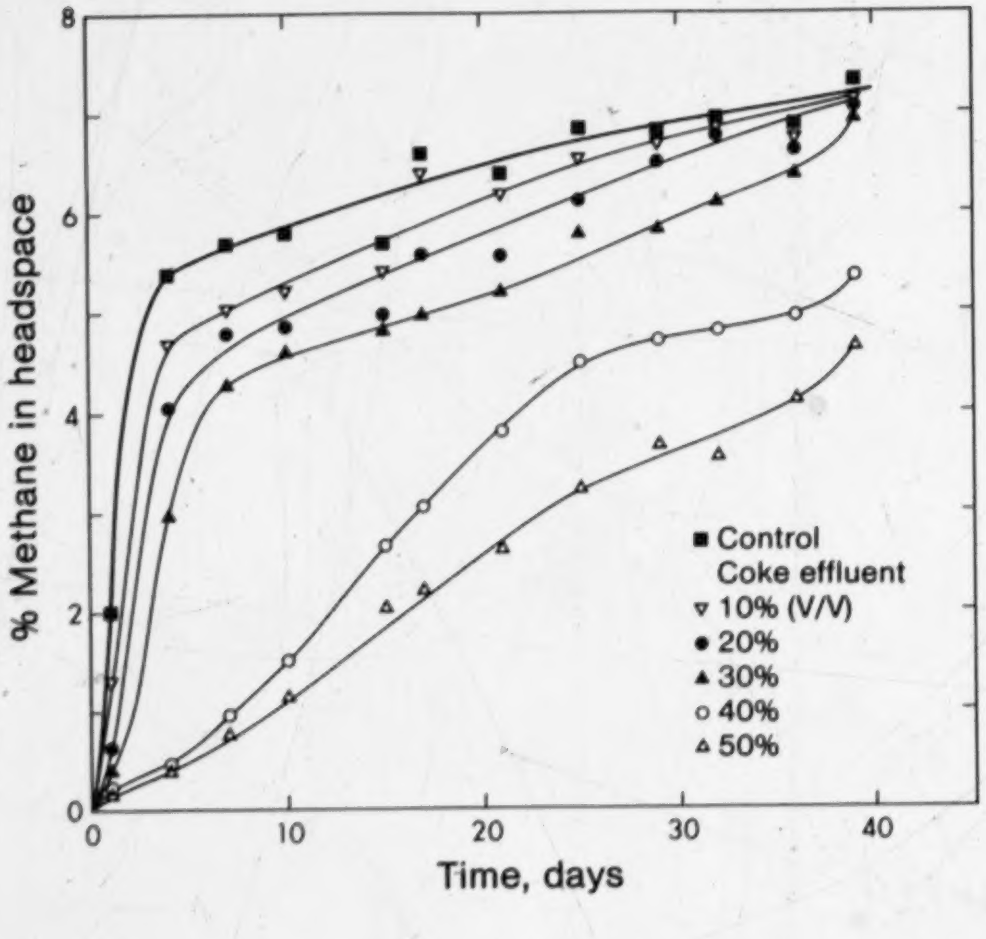


Figure 8.2 Methane production in batch cultures containing various concentrations of coke effluent - with VOA supplementation.

in the controls.

Cyanide concentrations were not measured during this study. However, based on the data in Table 8.1, the cyanide concentrations in the coke effluent-containing batch cultures would have been 0.83, 1.7, 2.5, 3.3 and 4.2 mg/L. Yang et al. (1980) studied cyanide inhibition in acetate enriched methanogenic cultures over a concentration range of 0.5 to 10 mg/L. Their batch culture work showed that given sufficient time, the methane concentration in each of the cyanide containing cultures reached that of the control. The higher the cyanide concentration, the longer the acclimation time. For example, with cyanide present at 2.5, 5, and 10 mg/L, lag periods of 9, 18 and 24 days, respectively were observed prior to methane production. This general trend was apparent with the coke effluent study (Figure 8.2) although the extent of inhibition was not as great as observed by Yang et al. (1980). Incubation of these batch cultures was not continued long enough to determine whether methane produced by the 40% and 50% wastewater cultures would reach that found in the control. These results suggest that cyanide may be partly responsible for the inhibition observed, but they cannot indicate that it is the sole cause.

8.2.2.2 H-coal Effluent

Batch cultures challenged with dilutions of H-coal effluent ranging from 2% to 15% were studied and Figure 8.3 summarizes the methane concentrations found in the cultures

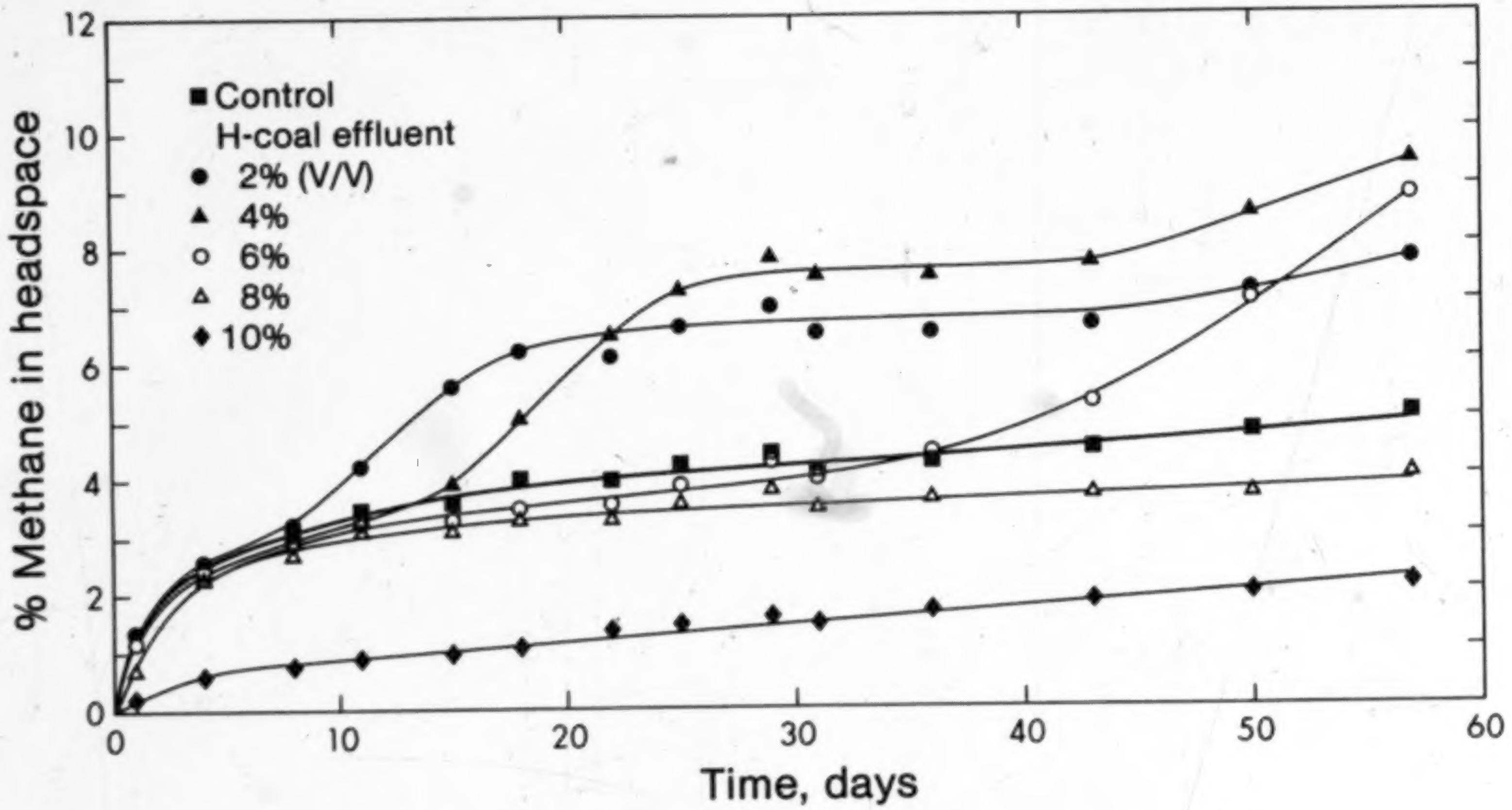


Figure 8.3 Methane production in batch cultures containing various concentrations of H-coal effluent - without VOA supplementation.

containing 2% to 10% effluent over a 57 day incubation period. At concentrations greater than 10%, the methane production was inhibited to the same extent as in the 10% effluent cultures. Consequently the results for the 12% and 15% effluent cultures were not plotted.

Three concentrations of H-coal effluent gave enhanced methane levels. These were 2, 4 and 6% which correspond to 152, 304, and 456 mg/L phenolics, respectively (based on data in Table 8.2). As observed earlier with single fermentable phenolics, higher concentrations of H-coal effluent (and therefore higher concentrations of phenolics) produced longer acclimation times. With 2% H-coal effluent, the acclimation time was 12 days; with 4% effluent, it was 16 days; and with 8% effluent it was 43 days.

Batch cultures containing 8% H-coal effluent showed varying responses depending upon the domestic sludge sample used for inoculation. In the case shown in Figure 8.3, methane concentrations in these cultures were only slightly less than the control values, while in other experiments with 8% H-coal effluent (data not presented), there was little or no methane production.

After 66 days incubation, the supernatant in a 4% H-coal effluent culture was analyzed by GC for phenolics. Figure 8.4 compares the resulting chromatogram with that obtained from the same culture at the time of inoculation (day zero). The selective loss of the phenol and m/p-cresol peaks was observed leaving mainly o-cresol and presumably

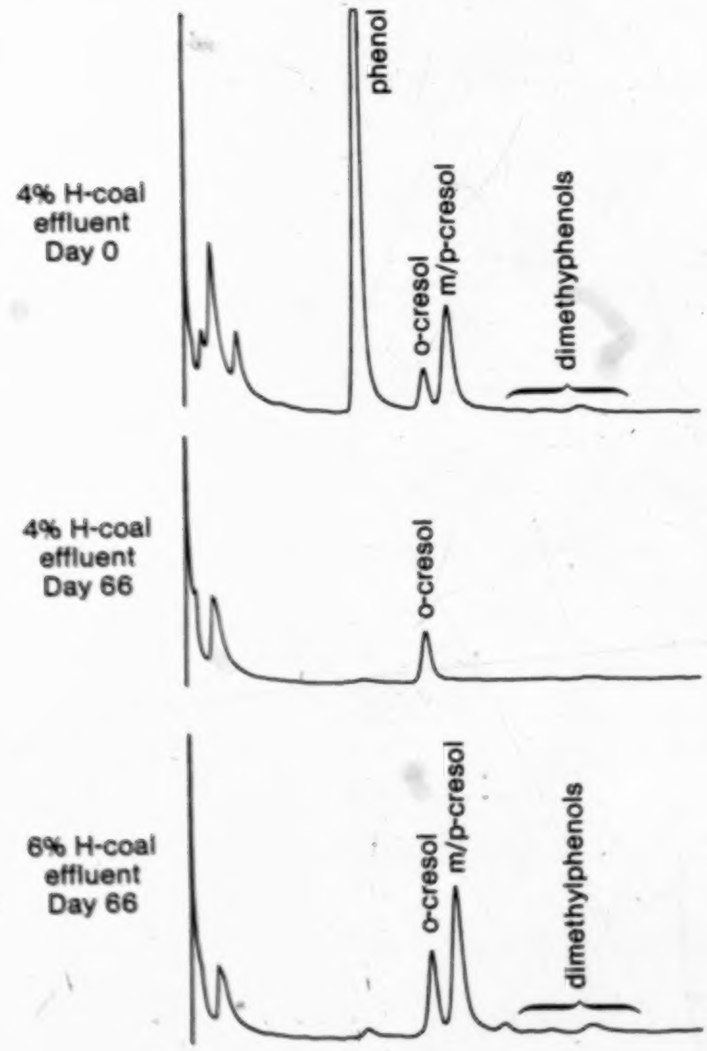


Figure 8.4 Substrate analyses of batch cultures containing H-coal effluent at various times.

the dimethylphenols which were barely detectable. This result is comparable to that in Figure 7.3 which showed the selective loss of phenol and p-cresol from a synthetic mixture of phenolics.

At the time that the results shown in Figure 8.4 were obtained, there had been no reports in the literature indicating that m-cresol was degradable under anaerobic conditions. Furthermore, the presence of m-cresol in the H-coal effluent had not been verified because of the overlap in m-cresol and p-cresol GC retention times. The observed loss of the m/p-cresol peak prompted the use of a different GC method to determine whether both isomers were present. As well, synthetic phenolic mixtures were tested to verify the degradability of m-cresol (Table 7.2). The analysis with the Carbopack C/0.1% SP-1000 GC column showed that m-cresol was present in the H-coal effluent. Thus m-cresol degradation was occurring anaerobically in the dilutions of the industrial wastewater.

During the early studies with H-coal effluent in batch cultures, the extent of phenolic degradation was monitored by comparing changes in the ratios of the area of a given peak to that of o-cresol rather than measuring the absolute concentration of each compound. Since there have been no reports in the literature of o-cresol being degraded under anaerobic conditions and there was no such evidence gathered in this study, o-cresol served as a convenient "internal standard" in the H-coal effluent cultures. Based on the

H-coal effluent data in Table 8.2, the ratio of m/p-cresol to o-cresol was 2.81 and the ratio of m-cresol to o-cresol was 2.09.

The complete loss of the m/p-cresol peak was not always observed in these batch cultures. Often the m/p-cresol to o-cresol ratio decreased to near 2 suggesting the removal of p-cresol but not m-cresol. In some cases the ratio decreased to zero and the m/p-cresol peak disappeared while in other cases the ratio remained constant. For example, the bottom chromatogram in Figure 8.4 shows the phenolics left after 66 days in a culture which contained 6% H-coal effluent. Although phenol had been removed, the m/p-cresol peak remained. Another analysis of this culture after a total of 78 days incubation gave a m/p-cresol to o-cresol ratio of 2.06. This value was very close to the 2.09 ratio expected if only m-cresol remained. Thus it is likely that only p-cresol had been degraded.

Data gathered from these H-coal effluent cultures clearly show that m-cresol can also be selectively removed from a complex phenolic wastewater along with phenol and p-cresol. They also show that m-cresol is the least susceptible to degradation in that the longest acclimation time is required prior to its loss from the cultures. The ability of batch cultures to degrade m-cresol is far more variable than is the case with phenol and p-cresol. Furthermore, cultures which ferment phenol and p-cresol do not always remove m-cresol from the phenolic mixture.

The addition of varying dilutions of H-coal effluent to VOA-supplemented cultures showed increased inhibition as the H-coal effluent concentration increased (Figure 8.5). On day 4, the cultures containing 2% H-coal had produced only 62% of the methane found in the control cultures while the cultures containing 4% and 6% effluent produced about 40% of the control value. The 8% effluent culture produced very little methane. The inhibition observed with the three lower concentrations did not persist and methane production in excess of that in the control was observed in the 2% and 4% effluent cultures after acclimation times of 28 and 34 days, respectively. By day 54, the methane concentrations in the 6% effluent cultures had reached those of the control cultures and there was a slight increase in methane levels found in the 8% effluent cultures.

These results indicate that at the H-coal effluent concentrations which are degradable in batch cultures (<6% V/V) there are components in the wastewater that retard, but do not stop, the conversion of VOAs to methane.

8.2.3 Batch Cultures Receiving Ether-extracted Wastewaters

Immediately after ether extraction of each wastewater, 3 µL aliquots were injected into the GC to determine the efficiency of phenolic removal. The presence of any phenol was masked by an extremely large peak of diethyl ether which remained dissolved in the aqueous phase. After remaining at room temperature overnight, the loss of the ether from the

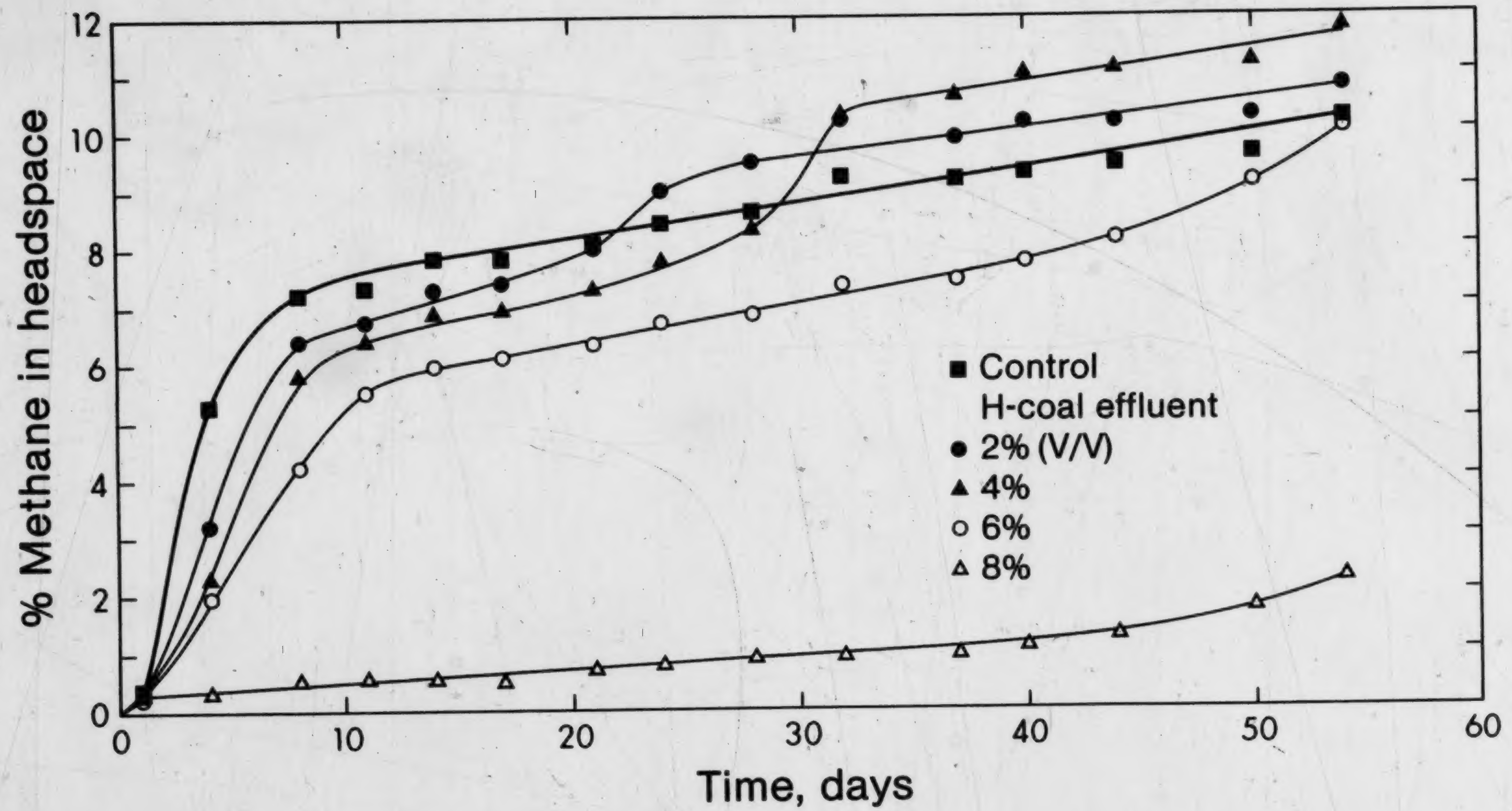


Figure 8.5 Methane production in batch cultures containing various concentrations of H-coal effluent - with VOA supplementation.

extracted effluent was observed by subsequent GC analyses. These analyses showed that >99.8% of the phenol had been removed from both the coke effluent and the H-coal effluent. Phenol was used as the indicator of extraction efficiency because it could be easily analyzed and it was clearly the predominant compound, accounting for nearly 50% of the organic carbon in each wastewater.

This extraction was intended to remove the majority of the organic compounds from the wastewater while leaving the inorganic ions in the aqueous phase. Various dilutions of the aqueous phase were then added to batch cultures to test for inhibition of phenolic degradation and/or methane production. Results from these experiments would then indicate whether the inorganic aqueous matrix or the extractable organic component of the wastewater was the cause for observed inhibition with the unextracted wastewater.

8.2.3.1 Extracted Coke Effluent

The methane production measured in the batch cultures which received varying dilutions of extracted coke effluent in non-VOA-supplemented medium is shown in Figure 8.6. One set of control cultures contained no phenol while the other set of controls and all of the test cultures contained 200 mg/L phenol. Neither set of controls received extracted coke effluent. As observed with the unextracted coke effluent (Figure 8.1), during the early stages of incubation the amount of methane produced decreased as the concentration of

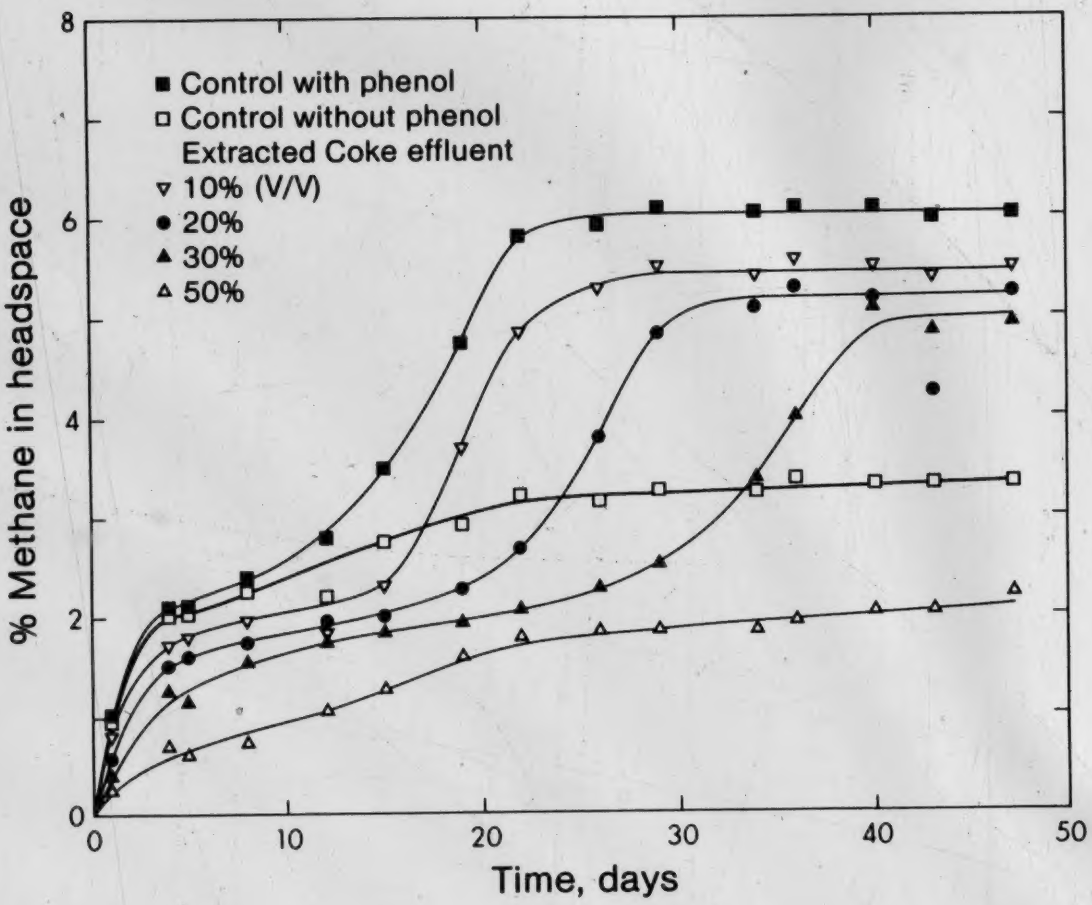


Figure 8.6 Methane production in batch cultures containing various concentrations of extracted coke effluent - without VOA supplementation.

extracted coke effluent increased. The mean methane evolution from the cultures containing 40% extracted coke effluent was the same as that observed with the 50% cultures and therefore the former set of data was not included in Figure 8.6. Complete phenol degradation and corresponding methane formation were observed in cultures which contained 10%, 20% or 30% extracted coke effluent. In the 40% and 50% extracted coke effluent cultures, methane generation was below that in the phenol-free controls and there was no loss of phenol.

Increased concentrations of extracted coke effluent in the batch cultures, also corresponded to increased time required to observe methane concentrations in excess of the non-phenol-containing control (Figure 8.6). These times were 12, 19, 26 and 36 days, respectively for cultures containing 0%, 10%, 20% and 30% extracted coke effluent. The amounts of methane produced by the three cultures containing extracted coke effluent were significantly less ($P<0.05$) than the amount formed in the control culture which contained phenol. However, this effect was not observed in the parallel series of cultures which were supplemented with VOAs (Figure 8.7).

The extraction of the coke effluent appears to have slightly reduced its inhibition of the conversion of VOAs to methane. Considering the range of doses in Figure 8.7, after one day incubation, the 10% culture had produced 84% as much methane as the control while the 50% culture had produced 23% as much as the control. The corresponding results from

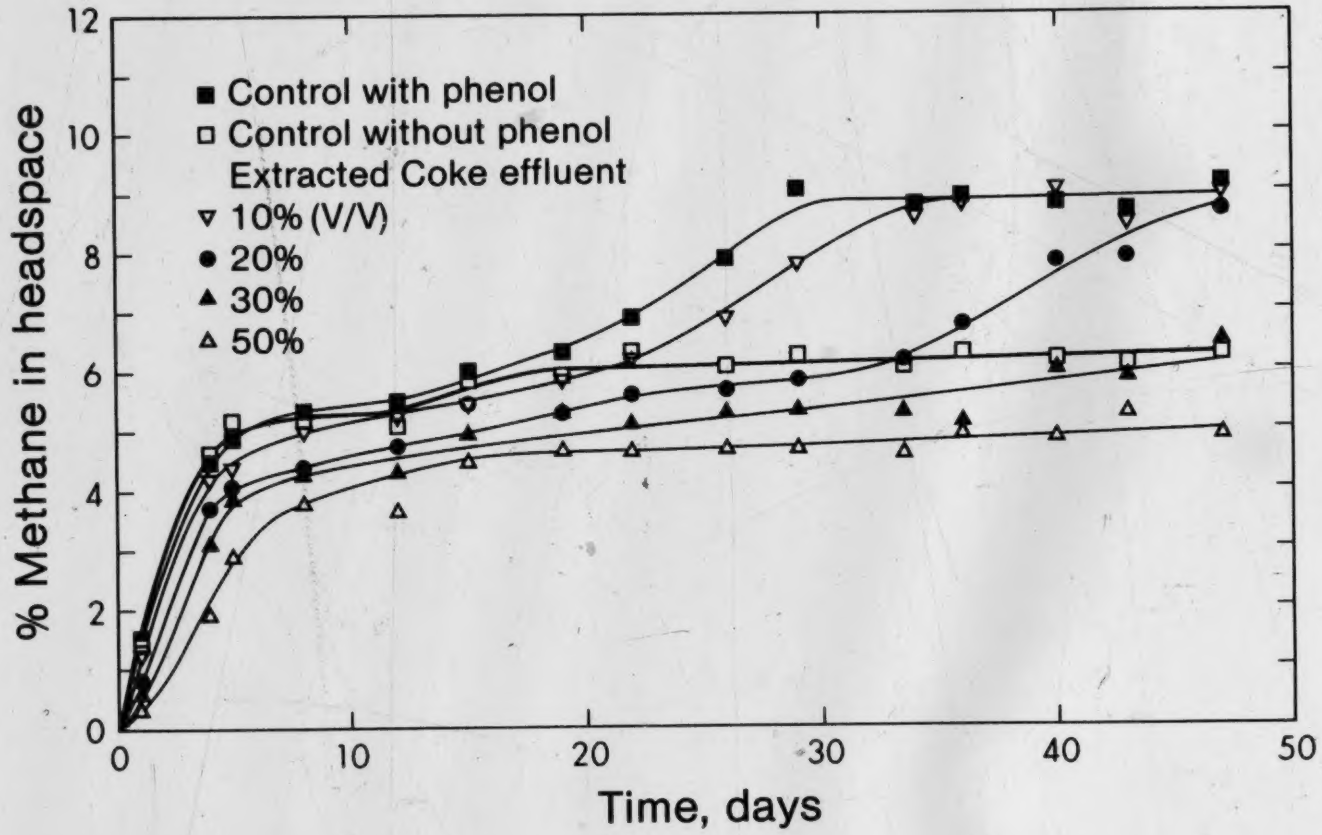


Figure 8.7 Methane production in batch cultures containing various concentrations of extracted coke effluent - with VOA supplementation.

the addition of unextracted coke effluent (Figure 8.2) indicated only 65% as much methane in the 10% cultures (compared to the control), and only 8% as much in the 50% cultures. The methane concentrations in the 10% and 20% coke effluent cultures reached that of the phenol-containing control after 34 and 47 days, respectively (Figure 8.7). By the end of the 47-day incubation period, the mean methane concentration in the 30% cultures reached that of the control without phenol, while those of the 40% (data not shown) and 50% cultures were still significantly less ($P<0.05$) than the control value.

The exhaustive diethyl ether extraction of the coke effluent did not greatly reduce its inhibitory effects on the conversion of phenolics to methane. The test cultures could tolerate the extracted aqueous phase until its concentrations reached >30% (V/V) in the batch solution. At levels above that, no loss of phenol was observed over the 47-day incubation periods of the batch cultures. The same results were found when the unextracted coke effluent was added to batch cultures indicating that non-extractable components - rather than the extractable organics - were responsible for the inhibition observed. The observations are consistent with cyanide toxicity, although they do not specifically attribute the inhibition of the methanogenic consortium to this anion.

8.2.3.2 Extracted H-coal Effluent

When the ether-extracted H-coal effluent was added to batch cultures in concentrations up to 25% (V/V), no inhibition of methane production nor phenolic degradation was observed. The fermentable phenolic substrate added to the batch cultures was phenol at 200 mg/L. Methane production in excess of the non-phenol-containing control cultures was observed after a suitable acclimation period. Increased concentrations of extracted H-coal effluent corresponded to increased methane production. These results suggested that some readily fermentable substrates were not extracted by ether from the H-coal effluent or that a small residual of diethyl ether dissolved in the aqueous phase might be converted to methane. Various volumes of diethyl ether were injected into batch cultures to determine whether this compound could be fermented to methane. No enhanced methane production was observed.

Ho et al. (1976) and Cleland et al. (1979) reported acetic acid in coal conversion wastewaters. Consequently the H-coal effluent was analyzed for VOAs and it was found to contain 280 mg/L acetic acid, 160 mg/L propionic acid, 50 mg/L butyric acid and 35 mg/L valeric acid. Analysis of the ether-extracted H-coal effluent showed essentially the same concentrations of VOAs as in the unextracted wastewater. Thus, the ether extraction at pH 7 did not remove these compounds so they provided a source of readily fermentable substrates for the methanogenic cultures.

The results from the first series of batch cultures indicated that the ether-extracted H-coal effluent was not inhibitory to the anaerobic process at concentrations ≤25%. Thus a second series containing higher concentrations was established.

When the extracted H-coal effluent was added at concentrations of 25% to 50% (V/V), the trend to higher methane production was again observed. Figure 8.8 shows the methane production from batch cultures which received 25%, 35% and 50% (V/V) either extracted H-coal. Two control cultures containing no extracted H-coal effluent were also monitored during this experiment. One contained 200 mg/L phenol while the other had no added phenol. By day 2, the methane concentrations in the extracted H-coal effluent cultures were significantly higher than those in the controls because of the presence of VOAs. The proportionally higher concentrations of methane that occurred with higher amounts of extracted H-coal effluent are evident between days 5 and 13. Using the 25% extracted effluent as the basis for comparisons, there were 1.4 and 2 times more extracted effluent added to the 35% and 50% cultures, respectively. Based on the concentrations of methane in excess of the control values on day 9 there was 1.3 times more methane in the 35% culture and 1.8 times more in the 50% culture than in the 25% culture.

After 16 to 20 days incubation (Figure 8.8), all of the phenol-containing cultures began to produce methane at

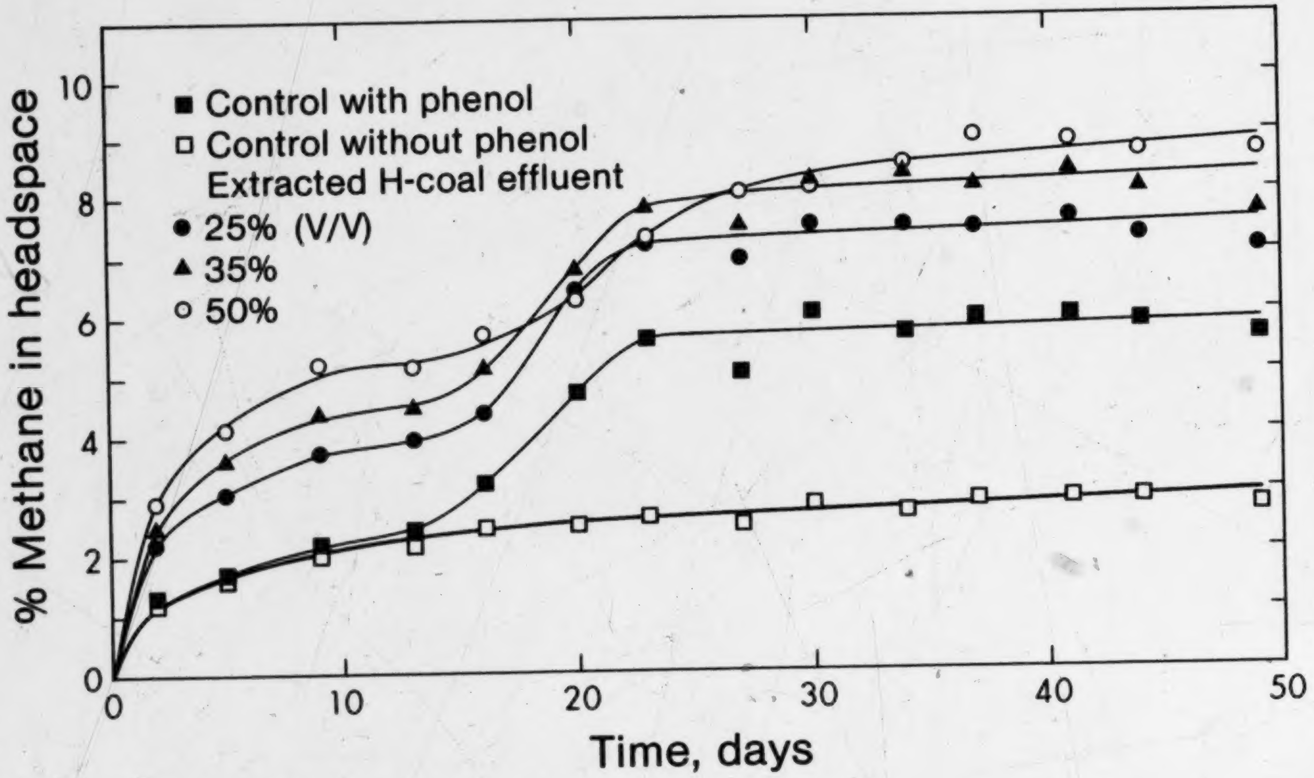


Figure 8.8 Methane production in batch cultures containing various concentrations of ether extracted H-coal effluent.

nearly the same rate. Thus at concentrations up to 50% (V/V), the non-extractable portion of the H-coal effluent was not inhibitory to either the methanogenic or non-methanogenic populations in the sludge. This wastewater exhibits a different effect than the extracted coke effluent which was extremely inhibitory to phenolic degradation and VOA fermentation when present at ≥40% (V/V).

8.2.4 Cultures Receiving Reconstituted H-coal Effluent

Batch cultures which received H-coal effluent at ≥8% (V/V) could not degrade the phenolics therein and usually exhibited almost total inhibition of methane formation (Figures 8.3 and 8.5). The total phenolic concentrations in the 8% and 10% H-coal effluent cultures were approximately 608 and 760 mg/L, respectively (based on the data in Table 8.1). It is unlikely that the phenolics alone were responsible for this severe inhibition since the methane production from cultures which received up to 1 200 mg/L phenol (Figure 5.4) or 600 mg/L p-cresol (Figure 5.6) was only slightly inhibited. Similarly, when synthetic mixtures of phenolics were added to batch cultures (Table 7.1), the fermentable compounds were often degraded in the presence of total phenolic concentrations near 700 mg/L (e.g. Cultures B and E, trial 1). In the case of culture B, trial 2 (Table 7.1), there was no degradation of phenol and p-cresol (total phenolic concentration of 744 mg/L) but the methane production was only slightly less than in the non-phenolic-containing control cultures.

The results from the extracted H-coal effluent batch cultures (Figure 8.8) clearly showed that the inorganic components of this wastewater were not causing the inhibition observed in the unextracted H-coal effluent batch cultures. Thus, it appeared that some ether-extractable component(s), presumably organic compound(s), is (are) responsible for the inhibition. This wastewater contains a variety of other aromatic compounds including quinoline, isoquinoline, methylquinolines, naphthalene, methylnaphthalenes, and fluorene. These are all present at concentrations <1 mg/L, except 8-methylquinoline which was present at 6.6 mg/L (Dearborn, personal communications). Aniline and 2,4-dinitrophenol were also present at 64 and 107 mg/L, respectively. Undoubtedly other organic compunds are present but there are no other data available on this wastewater.

In order to test the hypothesis that extractable organics were inhibitory, this experiment sought to remove all extractable organic materials from the H-coal and then to add back the phenolics to their original concentrations in the extracted aqueous phase. This reconstituted H-coal effluent was then tested in batch cultures to determine whether the mixed phenolics or some other organics were the cause of the inhibition.

During the qualitative and quantitative analysis of the H-coal, two minor peaks occurred in the chromatograms which could not be matched with the retention times of any of the authentic alkyl phenolic standards on hand. One peak eluted after phenol and before o-cresol and accounted for approximately 0.3% of total peak area on the chromatogram. The second unidentified compound eluted after m/p-cresol and before 2,6-dimethylphenol (the earliest eluting isomer) and accounted for about 0.4% of the total peak area. Given their uncertain identity, these minor constituants could not be similulated in the reconstituted H-coal.

The 2,4- and 2,5- isomers of dimethylphenol were not resolved by the GC method used and their retention time matched that of one of the peaks in the H-coal effluent. Arbitrarily, the 2,5- isomer was chosen to be added to the reconstituted H-coal effluent.

The reconstituted H-coal effluent was added to batch cultures in varying concentrations up to 16% (V/V). Methane production from selected concentrations are shown in Figures 8.9 and 8.10. To validate this approach, the effects of adding 2% and 4% reconstituted H-coal effluent to batch cultures were compared to those caused by adding 2% and 4% unextracted H-coal effluent. The resulting methane concentrations observed from this part of the experiment are shown in Figure 8.9. In all cases, methane was produced in excess of the levels found in the control cultures. On each of the last three sampling times (days 41, 44 and 49), there was no significant difference ($P<0.05$) between the methane concentration produced from the 2% H-coal effluent and that from

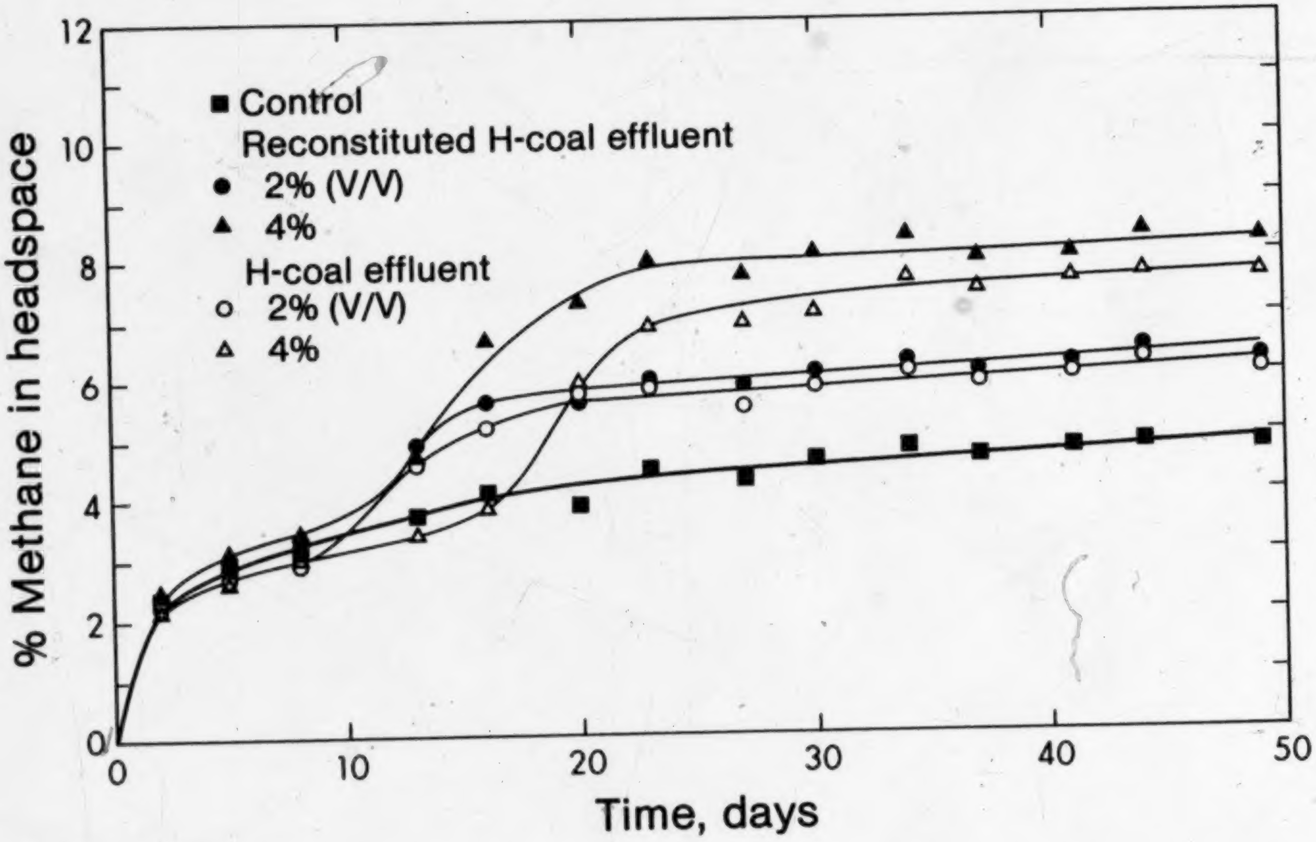


Figure 8.9 Methane production in batch cultures containing either H-coal effluent or reconstituted H-coal effluent.

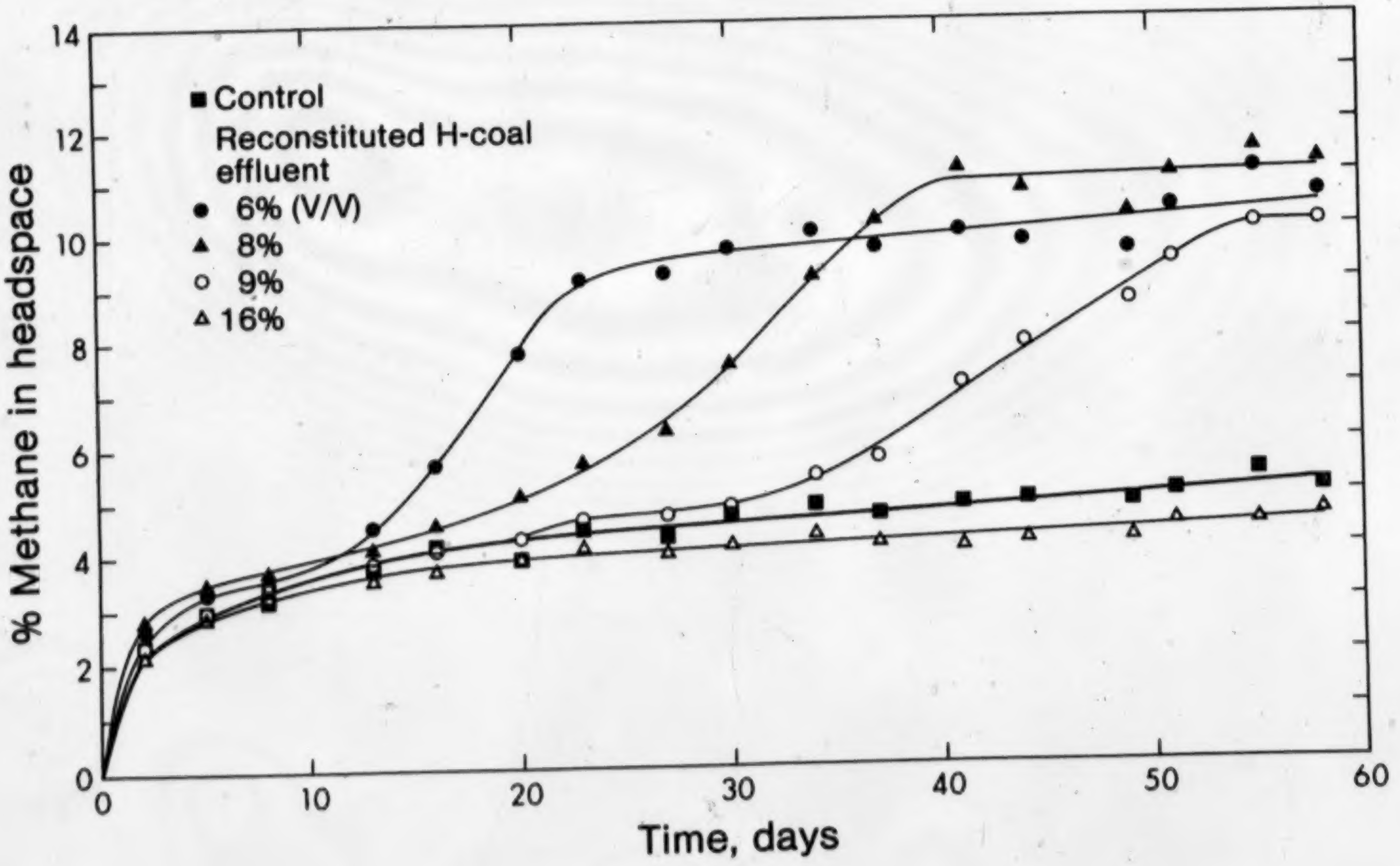


Figure 8.10 Methane production in batch cultures containing various concentrations of reconstituted H-coal effluent

the 2% reconstituted H-coal effluent. The same was true for the 4% H-coal effluent and 4% reconstituted H-coal effluent.

There was, however, a noticeable difference in the acclimation times of the two 4% cultures. The reconstituted H-coal effluent culture required between eight and 13 days while the H-coal effluent culture required between 16 and 20 days. This marked difference suggested the presence of an ether-extractable component in the H-coal effluent which caused mild inhibition even when diluted to 4% (V/V) in the batch cultures.

A comparison of the methane production from cultures containing reconstituted H-coal effluent (Figure 8.10) with the data from cultures with unextracted H-coal effluent (Figure 8.3) clearly shows that the inhibition observed with the unextracted H-coal effluent is not due solely to the phenolic content. The highest concentration of unextracted H-coal effluent which was amenable to anaerobic degradation was 6% (V/V) and the cultures required 43 days to acclimate (Figure 8.3). With 6% reconstituted H-coal effluent the acclimation time was only 16 days (Figure 8.10). After 20 days incubation, methane production above controls occurred in the cultures containing 8% reconstituted H-coal effluent (equivalent to a total phenolics concentration of 596 mg/L). Two of the three replicate cultures containing 9% reconstituted H-coal effluent (671 mg/L phenolics) began to degrade these compounds by day 34. Enhanced methane production started in the third culture after 49 days but the rate of gas formation in this culture was much slower than in the other two. By day 55, enhanced methane levels were detected in the cultures containing 10% reconstituted H-coal effluent (746 mg/L phenolics). The latter data were not included in Figure 8.10 to avoid congestion.

The highest concentration of reconstituted H-coal effluent tested in batch cultures was 16% (V/V) which contained 1 194 mg/L phenolics. Although there was no enhanced methane production from this concentration, the methane produced was not significantly less than that found in the control cultures (Figure 8.10). With unextracted H-coal effluent there had been marked inhibition of methane production relative to controls at both 10% (V/V) (Figure 8.3) and 8% (V/V) (Figure 8.5).

Phenolic analyses done on day 57 for the cultures containing ≤8% reconstituted H-coal effluent showed that phenol had been removed from each culture. Also by that time, the m/p-cresol peak had been removed from most cultures which received ≤6% reconstituted H-coal effluent. The exception was one of the triplicate cultures containing 2% reconstituted H-coal effluent in which only p-cresol had been degraded. There appeared to be little degradation of the m- and p- isomers in the 8% cultures.

The results from the experiment with the reconstituted H-coal effluent show that ether-extractable compound(s), other than the phenolics, is (are) responsible for the previously observed inhibition in cultures containing unextracted H-coal effluent. These results also agree with data from cultures receiving synthetic mixtures of phenolics given in chapter 7. For example, cultures given six phenolics at total concentrations near 700 mg/L could selectively degrade the fermentable phenols (Figure 7.2). Methane production in excess of the controls was observed with cultures given 9% and 10% reconstituted H-coal effluent which corresponded to 671 and 746 mg/L phenolics respectively. Many of the cultures containing reconstituted H-coal effluent were able to completely remove the added m-cresol, further verifying that this compound is susceptible to anaerobic degradation.

8.2.5 Ultimate Methane Production from H-coal Effluent

Two sets of triplicate cultures containing 1.0 mL aliquots of H-coal effluent (2% V/V) and corresponding controls were established and monitored for absolute methane production. In both cases, the maximum methane levels were reached after 20 days incubation. However these cultures were allowed to incubate longer with occasional methane measurements performed. The first set incubated for a total of 40 days and the mean methane yield was found to be 7.1 mL (at STP) per mL H-coal effluent with a standard deviation of 0.7 mL. The second set incubated for 62 days and the mean methane yield was 6.1 mL (at STP) per mL H-coal effluent with a standard deviation of 1.1 mL. These means were found to be significantly different ($P<0.05$).

Based on the VOA content of the H-coal effluent and assuming 100% conversion of these compounds to methane, Buswell's equation predicts 0.23 mL methane (at STP) per mL H-coal effluent. Considering the ultimate methane yield per mg phenolic from Table 5.5 and the analysis of the phenolics in the H-coal effluent (Table 8.2), phenol and p-cresol should give 3.67 and 0.33 mL methane (at STP) per mL H-coal effluent, respectively. Assuming that 86% of the m-cresol carbon is converted to methane (i.e. the same proportion as p-cresol), it should account for 0.90 mL methane (at STP) per mL H-coal effluent. Thus, the total volume of methane expected from these seven compounds is 5.13 mL per mL H-coal effluent.

The results from both sets of batch cultures showed that more than 5.13 mL methane was produced from each mL H-coal effluent. These data suggest that other fermentable compounds were present in this effluent which were not detected nor quantitated by the GC methods used. Based on GC-mass spectrometry analysis, Dearborn Environmental Consultants report that catechol is present at about the same concentration as m-cresol and that resorcinol is found at nearly the same concentration as p-cresol (personal communications). Both of these phenolics are fermentable (Table 2.4).

Analyses of the culture supernatants showed that the first series of cultures completely removed the m/p-cresol peak, while in the second series, this peak remained. Over the incubation period, the peak area ratio of m/p-cresol to o-cresol decreased from 2.9 to 1.7 in the second series, suggesting that m-cresol remained undegraded. Since the amount of m-cresol present in these cultures should produce 0.9 mL of methane, the average volumes of gas produced in these two test series should differ by that value. The observed difference was 1.0 mL of methane which strongly suggested that m-cresol was not degraded in the second series of cultures.

9. FERMENTATION OF PHENOLICS IN SEMICONTINUOUS CULTURES

Data gathered from batch culture studies with individual and mixtures of phenolics have provided new insight into the anaerobic degradation of these compounds by methanogenic consortia. However, in order to use these microbial activities in the treatment of industrial phenolic wastewaters, they must be proven to persist in continuous operation over long periods of time. Semicontinuous (draw and feed) cultures were used to bridge the gap between batch and continuous culture methods. A simple modification of the Hungate serum bottle method was used to facilitate the transition from batch to semicontinuous culture.

The first semicontinuous culture to be established was one which received phenol in a mineral solution supplemented with vitamins. After determining that an active culture could be maintained for a long period of time, a p-cresol-degrading semicontinuous culture was studied. Finally, the treatability of the H-coal effluent was studied using six draw and feed cultures - each receiving a different loading rate.

9.1 Procedures

9.1.1 Culture Methods

Active cultures were enriched from domestic anaerobic sewage sludge. Prior to inoculating, each 158 mL serum bottle was flushed with O_2-free 30% CO_2 in N_2. Then 1 mL resazurin solution and 45 mL sludge were added. Appropriate aliquots of substrate phenolic solution and distilled water were added to give the desired final phenolic concentration in a total volume of 50 mL. Control cultures, devoid of phenolic substrate, were also inoculated. All cultures were incubated at 37°C and methane production and phenolic loss were monitored by the appropriate GC methods. When most of the phenolic substrate had been depleted, the headspace gas was exchanged to remove all the methane that had accumulated. The exchange procedure involved repeated evacuating and filling the headspace with O_2-free 30% CO_2 in N_2. After several such cycles, the methane concentration was measured by GC analysis to determine whether further flushing was required. When there was a negligible amount of methane left in the headspace gas, the pressure in the bottle was adjusted to ambient atmospheric pressure as outlined in section 5.1.5.

Figure 9.1 summarizes the draw and feed procedure. Cultures were typically incubated still and upright as shown in step 1 (although the H-coal fed cultures were incubated in an inverted position). After removal from the incubator and shaking, the bottle was allowed to stand inverted for approximately 20 min while the solids settled (step 2). Then a 27G x 3.2 cm needle fitted onto a glass syringe was inserted through the serum stopper and the appropriate volume of supernatant was withdrawn (step 3). The bottle was returned to its upright position and the appropriate volume

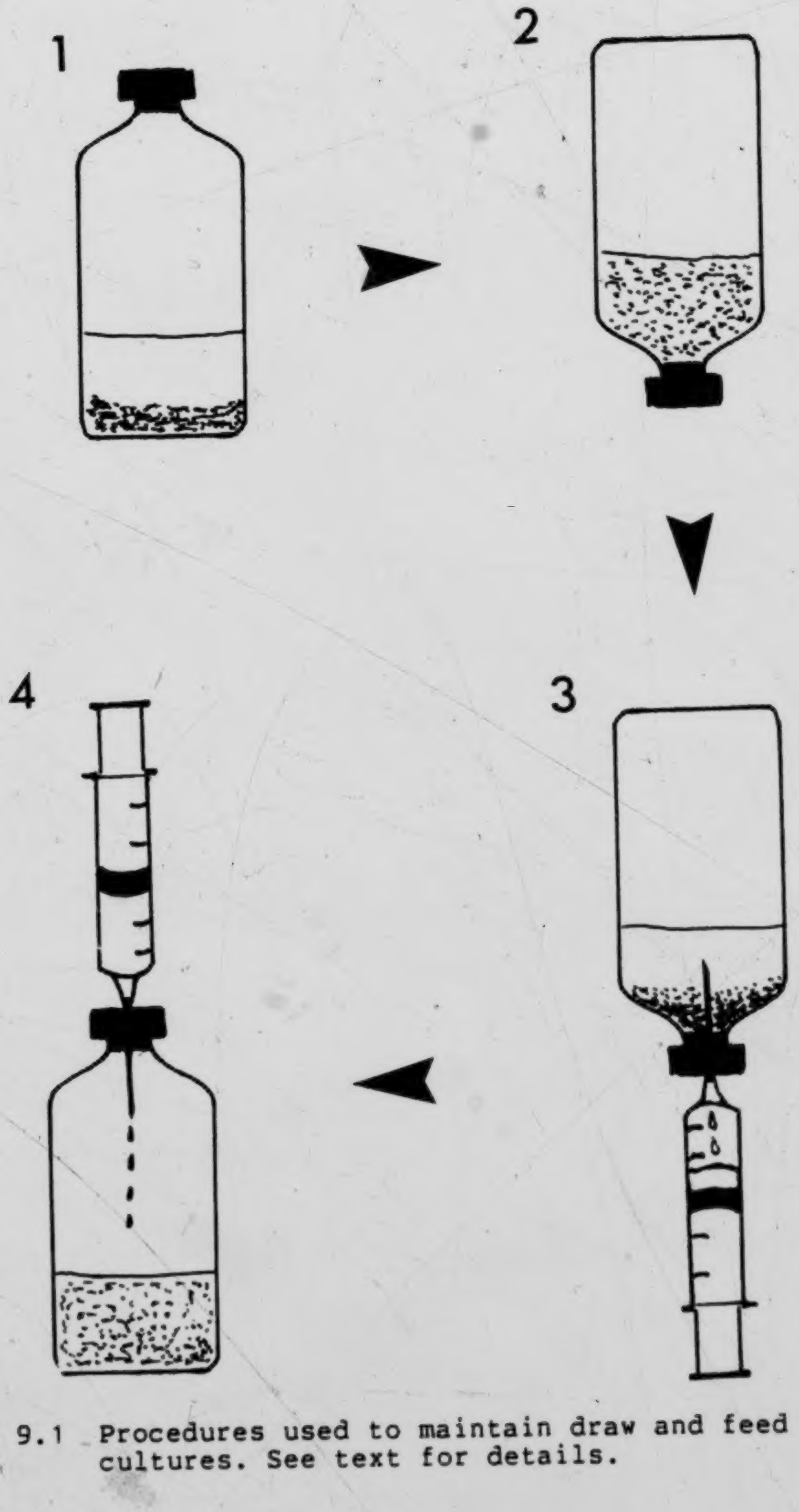


Figure 9.1 Procedures used to maintain draw and feed cultures. See text for details.

of feed solution was added (step 4). Finally the culture was returned to the incubator.

Absolute methane production was frequently determined using the gas measuring device described in section 3.2.3. After approximately 100 to 120 days of incubation, the butyl rubber stopper in each bottle was replaced. This precaution was taken to minimize the chance of gas leakage resulting from the scores of needle punctures through the serum stopper. As the stopper was removed, a flow of O_2-free 30% CO_2 in N_2 was immediately directed into the bottle to prevent O_2 from entering the culture. After a new stopper was inserted, the headspace gas was exchanged to remove all accumulated methane and the bottle adjusted to ambient atmospheric pressure. This operation typically marked time zero for another monitoring period. However, in the case of the semicontinuous cultures receiving H-coal effluent, the amount of methane in each culture bottle was determined just prior to replacing the serum stopper. The subsequent volumes of methane measured were then added to the amount of methane produced up to the time of the serum stopper exchange to provide a cumulative total methane record.

9.1.2 Feed Solution Composition

A stock solution of 3.3 mg KH_2PO_4, 6.4 mg NH_4Cl and 4.3 mg K_2SO_4 in 1 L distilled water was prepared. A 99 mL aliquot of this solution was combined with 0.1 mL Vitamin B solution, 1 mL mineral solution I, 0.1 mL mineral solution

II and 1 mL resazurin solution (see Table 3.1). After boiling for approximately 3 min, 0.57 g $NaHCO_3$ was added and the medium was cooled while bubbling with O_2-free 30% CO_2 in N_2 until the pH was near 7.

Suitable aliquots were dispensed into gassed serum bottles containing the desired weight of phenolic substrate or the desired volume of H-coal effluent. The bottles were sealed and those containing pure phenolics were autoclaved. Those containing H-coal effluent were immediately refrigerated. Just prior to use, sulfide solution (Table 3.1) was added to each bottle at the rate of 0.1 mL per 10 mL feed solution. Control feed solutions contained no phenolic or no H-coal effluent.

9.1.3 Cultures Receiving Pure Phenolic Substrates

An active phenol-degrading batch culture was enriched on 500 mg/L phenol and a p-cresol-degrading batch culture was enriched on 200 mg/L p-cresol. These were then maintained as individual semicontinuous cultures. One was fed a solution containing 500 mg/L phenol while the other was fed a solution containing 600 mg/L p-cresol. The draw and feed procedure was done on Mondays and Fridays when 8 mL and 6 mL aliquots, respectively, were exchanged giving a mean hydraulic retention time of 25 days.

9.1.4 Cultures Receiving Diluted H-coal Effluent

Six cultures were enriched on 2% (V/V) H-coal effluent along with three control cultures. After 15 days incubation, phenol had been removed from the enrichment cultures and the draw and feed procedure was started according to the scheme shown in Table 9.1. During the first 13 days of semicontinuous culture operation, the cultures were fed only five times to ensure a smooth start-up. From day 14 onward, all of the cultures were fed daily. On day 22, the headspace gas in each bottle was exchanged to remove all methane that had accumulated and absolute methane volumes were then monitored thereafter.

Starting on day 59, effluent samples from each culture, except A, were collected and frozen until a composite volume of 60 mL had been collected. These were then extracted with CH_2Cl_2 and analyzed using the Carbopack GC method (section 8.1.1.1) which can resolve the m- and p- isomers of cresol. This sampling time was designated "I". A second sampling time, designated "II", was started on day 92 and composite samples from all six cultures were analyzed for m- and p-cresol.

9.2 Results and Discussion

Studies of anaerobic digestion (Sanders and Bloodgood, 1965; Boone, 1982), inhibition of the anaerobic process (Sykes and Kirsch, 1972), and the biokinetics of anaerobic phenol degradation (Neufeld et al., 1980) have successfully

Table 9.1 Summary of semicontinuous cultures maintained on diluted H-coal effluent.

Culture designation	Draw and feed volume/day (mL)(1)	H-coal effluent in feed solution
A	2	2%
B	3	2%
C	4	2%
D	2	4%
E	3	4%
F	4	4%
Control A	2	0
Control C	4	0

(1)Fed to 50 mL culture

used draw and feed methods. In general, the laboratory scale apparatus has been quite large (1-10 L) and complex with a stirring mechanism, temperature control, gas collection device and sampling ports (Hawkes and Young, 1980).

The draw and feed experiments reported here were done using 158 mL serum bottles as simple reactors and was similar to the method used by Parkin et al. (1983). Because of their small size and simplicity, many cultures can be incubated in an ordinary laboratory incubator which allows a wide variety of test conditions to be evaluated simultaneously. This method for fermentability screening studies offers significant advantages over larger scale digesters. Gas production measurements were readily made with the apparatus described earlier (section 3.2.3) and small aliquots can readily be removed for GC analyses of the phenolic substrates. Although not routinely used in this study, continuous mixing can easily be attained by clamping the culture bottles onto a standard laboratory rotary shaker operating at any desired speed.

The draw and feed procedure using a syringe provides an easy method to establish and control any chosen hydraulic retention time. A mean cell residence time equal to that of the hydraulic retention time can be maintained by ensuring that the culture remains well mixed while the effluent is being withdrawn. Alternatively, a very long mean cell residence time (approximating an attached growth system) can be maintained. In this case, the biomass is allowed to settle prior to removing the clear supernatant with a hypodermic needle long enough to extend beyond the depth of the biomass. Any desired mean cell residence time between these two extremes can be attained by first removing an appropriate volume of supernatant and then removing an aliquot from the remaining culture while it is being completely mixed.

9.2.1 Cultures Receiving Pure Phenolic Substrates

The phenol-degrading semicontinuous culture was initially established to determine whether the serum bottle method could be used to maintain an active culture over a long period of time and to provide a source of inoculum of a phenol acclimated consortium. An average daily draw and feed rate of 2 mL/day was chosen for the 50-mL cultures. Care was taken not to remove biomass when the effluent was being removed in order to maintain the maximum number of cells in the culture.

To date, this semicontinuous culture has been maintained for 24 months. The biomass has had excellent settling properties and a clear effluent has been obtained. Although the microbial population in this culture has not been examined, the presence of facultative anaerobes was apparent based on the following observation. Upon removal of the supernatant, the effluent was essentially colourless in the syringe. When this was injected into an open test tube, the medium quickly oxidized and the redox indicator (resazurin) turned pink. When the tube was left undisturbed for approximately 20 min the liquid became colourless (reduced) because of the O_2 consumption by the facultative anaerobes. Although strict anaerobic techniques were used during the draw and feed procedure, these observations suggest that if a small amount of O_2 was inadvertently introduced into the culture, it would be quickly consumed, thus minimizing the chance of adversely affecting the strict anaerobes in the consortium.

After the phenol-degrading culture was well established, its ability to convert the substrate to methane was monitored by measuring the absolute methane production. Results of four experimental trials, each lasting between 108 and 115 days, are summarized in Figure 9.2. Initially the methane production of a control culture was monitored along with that of the phenol-degrading culture. However, the methane production in the control culture was found to be negligible and therefore only the phenol-degrading culture was monitored.

Figure 9.2 compares the observed methane production rate with two predicted rates. Line A shows the expected methane production if the conversion of phenol follows the stoichiometry suggested by Buswell and Muller (1952). Prediction line B was based upon the earlier batch culture experiments to determine the ultimate methane formation from phenol (section 5.2.4). Since the semicontinuous culture received an average of 1 mg phenol/day, line A has a slope

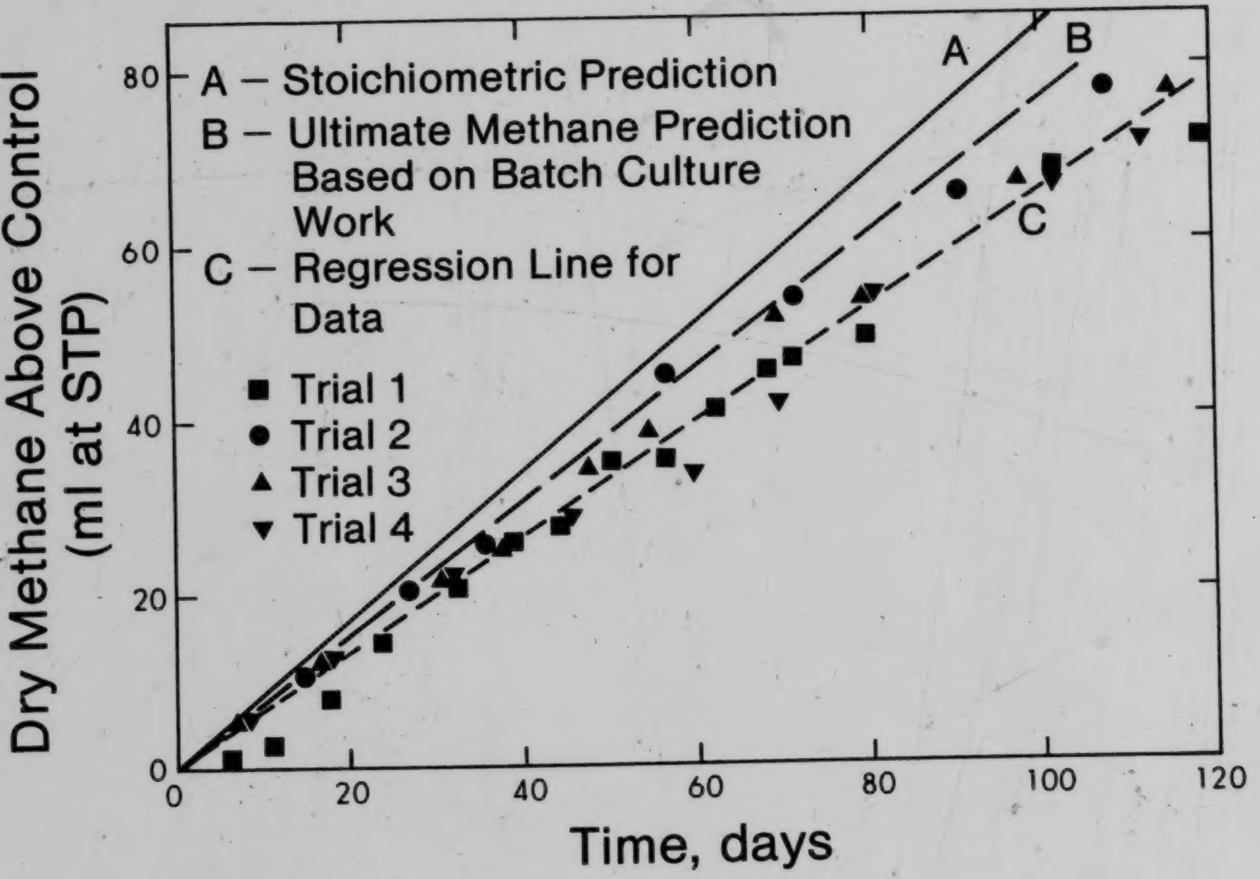


Figure 9.2 Methane production from phenol degradation in a semicontinuous culture. Experimental data compared to predicted values.

of 0.835 mL methane/day and line B has a slope of 0.75 mL methane/day. Linear regression analysis with the 41 measurements shown in Figure 9.2 gave a slope of 0.647 mL methane/day (r=0.989). As expected, the observed values agreed more closely to line B than to line A. The Student t-test was used to compare the slope of the observed methane production with that of line B (Ryan *et al*., 1976). These were found to be significantly different ($P>0.05$) with the observed rate being only 86% of the expected rate. The rates of the four individual trials were 0.61, 0.71, 0.65 and 0.62 mL methane/day, respectively. The rate from the second trial was not significantly less than the expected value (i.e. line B) although all of the others were less than expected. However, there was no apparent trend in the rates to indicate that the culture's activity was increasing or decreasing over the extended incubation period.

On several occasions after the culture had been fed on a Monday, phenol concentrations were determined three to four times per day until the substrate had disappeared. This provided a 96 h test period prior to the next feeding. Typically there was a lag of 5 to 15 h prior to a reduction of the phenol concentration. The substrate was usually depleted within 40 to 70 h after feeding. This would suggest that the culture had only 26 to 56 h to ferment all of the VOAs to methane prior to the next feeding. At the time these experiments were done, a method for VOA analysis had not been implemented and therefore no quantitative data are available. However, it is not likely that all of the VOAs produced from the aromatic substrate were converted to methane before the next feeding time. Removal of a fraction of these in the withdrawn effluent would account, in part, for the lower than expected methane production rate.

A 50 mL culture enriched on p-cresol was maintained as a semicontinuous culture for one 88-day period. It was fed an average of 2 mL/day (based on 2 feedings a week) of a solution containing 600 mg/L p-cresol. The methane production was monitored and is shown in Figure 9.3. The observed rate of methane evolution (line C) was 0.912 mL/day ($r=0.999$) and this was significantly less than the rate of 1.06 mL/day ($P>0.05$) predicted by Buswell's equation (line A). The observed rate was essentially identical to the 0.91 mL/day rate based on the ultimate methane produced in batch cultures (section 5.2.4).

The better agreement between the observed and expected rates in the p-cresol-degrading culture than those in the phenol-degrading culture would suggest that the former culture was much more active. Although not quantitatively determined, the p-cresol-degrading culture appeared to contain much more biomass than did the phenol-degrading culture. The nature of the biomass in the two cultures also differed. The phenol-degrading culture contained pin point, granular particles while the p-cresol-degrading culture contained larger flocculent material. Whether these different physical appearances were due to the nature of the

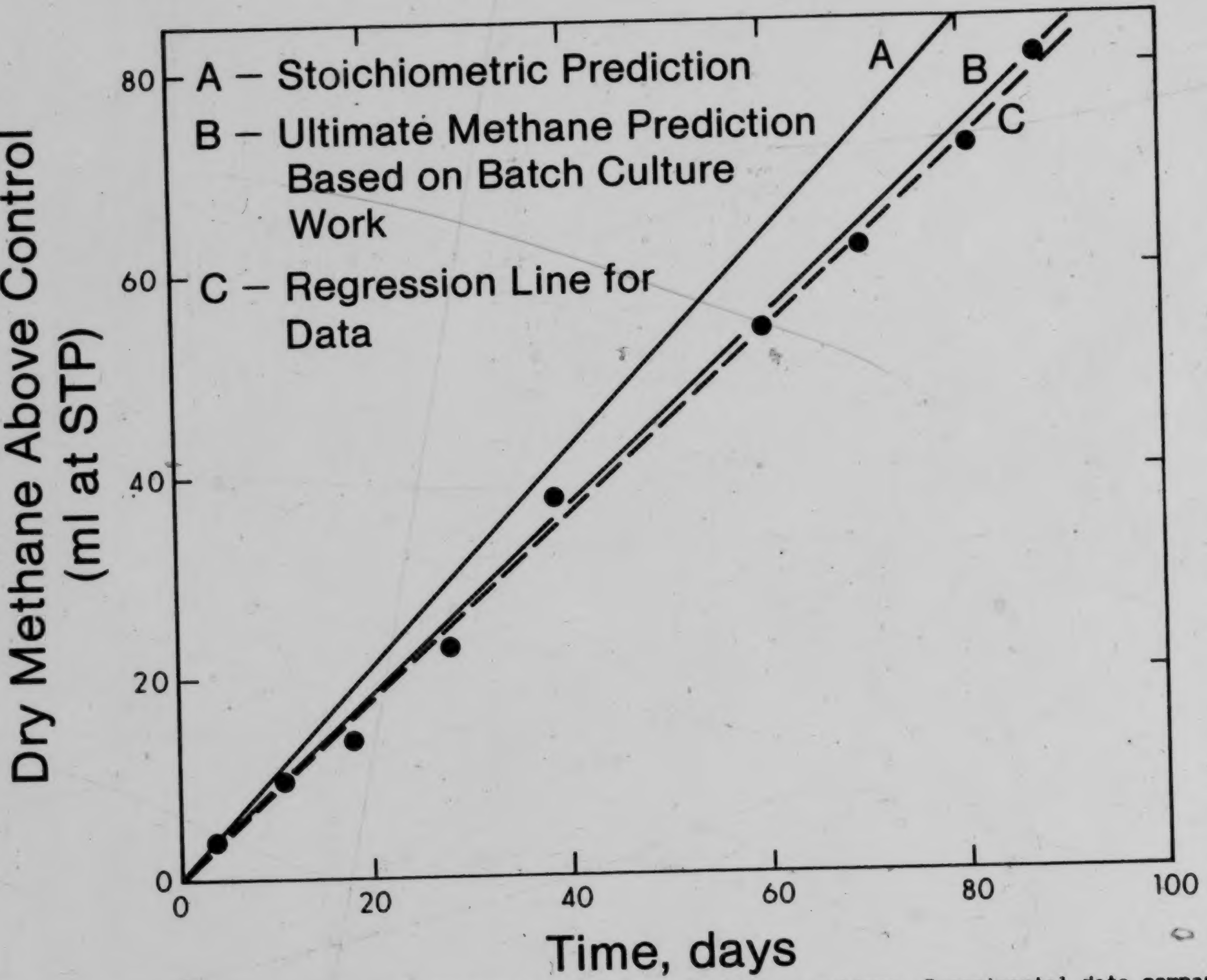


Figure 9.3 Methane production from p-cresol degradation in a semicontinuous culture. Experimental data compared to predicted values.

specific phenolic-degrading consortia or simply a result of the different domestic sludge samples used as inocula has not been determined.

The results from these two semicontinuous culture experiments clearly showed that methanogenic consortia could be maintained for long periods of time on either phenol or p-cresol in vitamin-supplemented, defined medium. They also demonstrate that the activities of such cultures can be monitored by measuring the absolute methane production rate and that rates based on ultimate methane production in batch cultures (rather than on Buswell's equation) serve as better measures of the culture's overall performance.

9.2.2 Cultures Receiving Diluted H-coal Effluent

Data obtained from batch culture experiments showed that dilutions of H-coal effluent were amenable to phenolic fermentation. However, the maximum concentration which was not inhibitory to the phenolic degradation process was 6% (V/V) and 8% usually inhibited methane production from other fermentable compounds in the sludge inoculum. Work with reconstituted H-coal indicated that some organic compound(s), other than phenolics, was (were) inhibitory at ≥8% (V/V) H-coal effluent. Thus, the maximum concentration which the H-coal effluent could reach in a semicontinuous culture was 6%. Higher concentrations would be inhibitory. Since the inhibitory compounds in H-coal effluent have been shown to completely stop the anaerobic process, it is unlikely that these compound would be biodegradable under these conditions. Thus the continual addition of a feed solution containing these compounds will result in their concentrations in the culture bottle eventually reaching those in the feed solution. This is analogous to the continuous (or step) input of a non-reactive tracer being injected into a complete mix reactor (Metcalf and Eddy, 1979). Therefore, the H-coal effluent must be diluted to 6% (V/V), or less, to maintain activity in semicontinuous cultures.

The batch cultures used for the preliminary enrichment contained 2% H-coal effluent. By day 15, phenol had been depleted and the m/p-cresol concentration was near 25 mg/L. If both of the unresolvable cresol isomers were present, the expected concentration would have been 33 mg/L while the expected concentration of m-cresol would have been 24.6 mg/L (from data in Table 8.2). Thus it was likely that the only fermentable compound that remained was m-cresol. At this time (day 15 of the batch culture), the draw and feed procedure was started and was designated as time zero for the semicontinuous cultures. By day 14, the phenol concentration in all six semicontinuous cultures was <2 mg/L (Figures 9.4-9.9) and the levels of m/p-cresol were undetectable in cultures A to E. At this time, culture F contained 11 mg/L m/p-cresol.

The concentration of o-cresol remained at a constant level near 11 mg/L in bottles A, B and C. Since o-cresol has not been found to be degradable under anaerobic conditions,

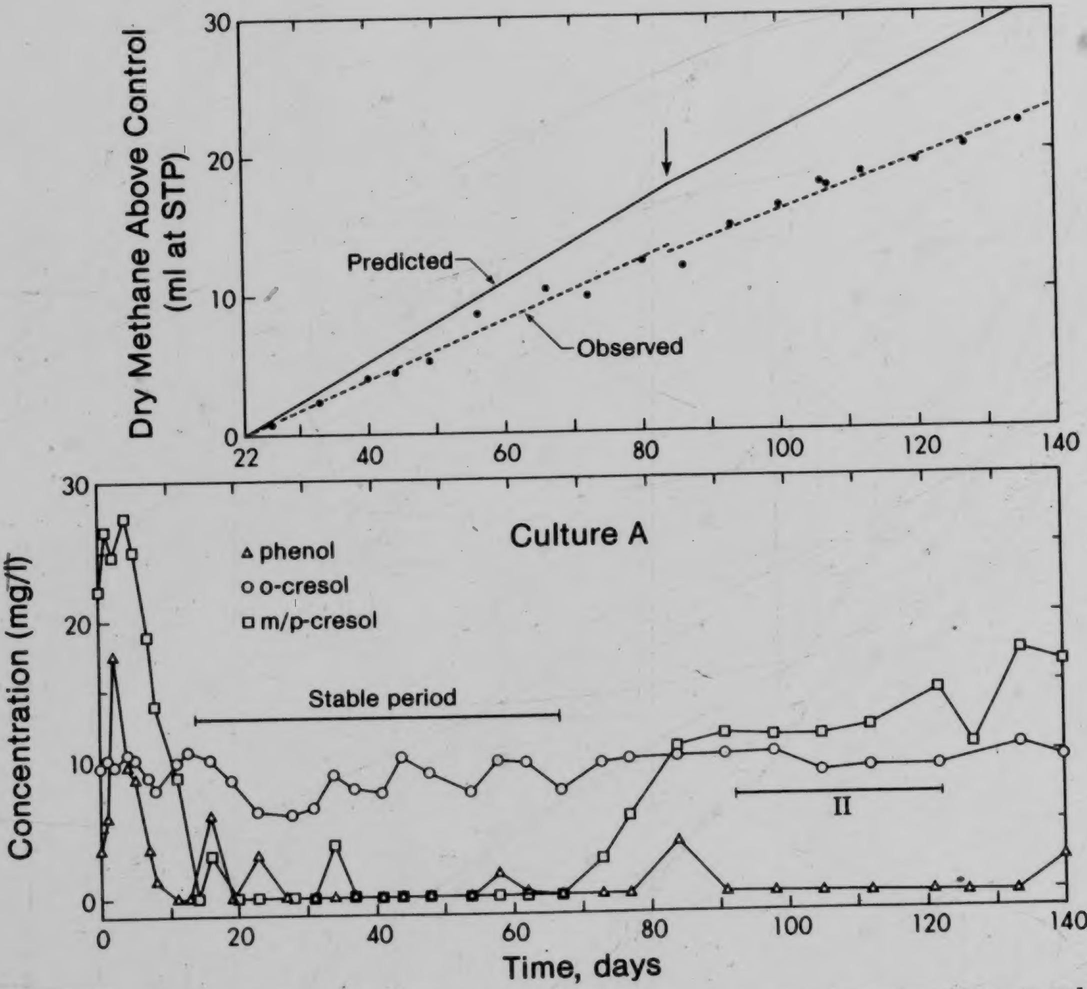


Figure 9.4 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 2 mL of 2% H-coal effluent each day.

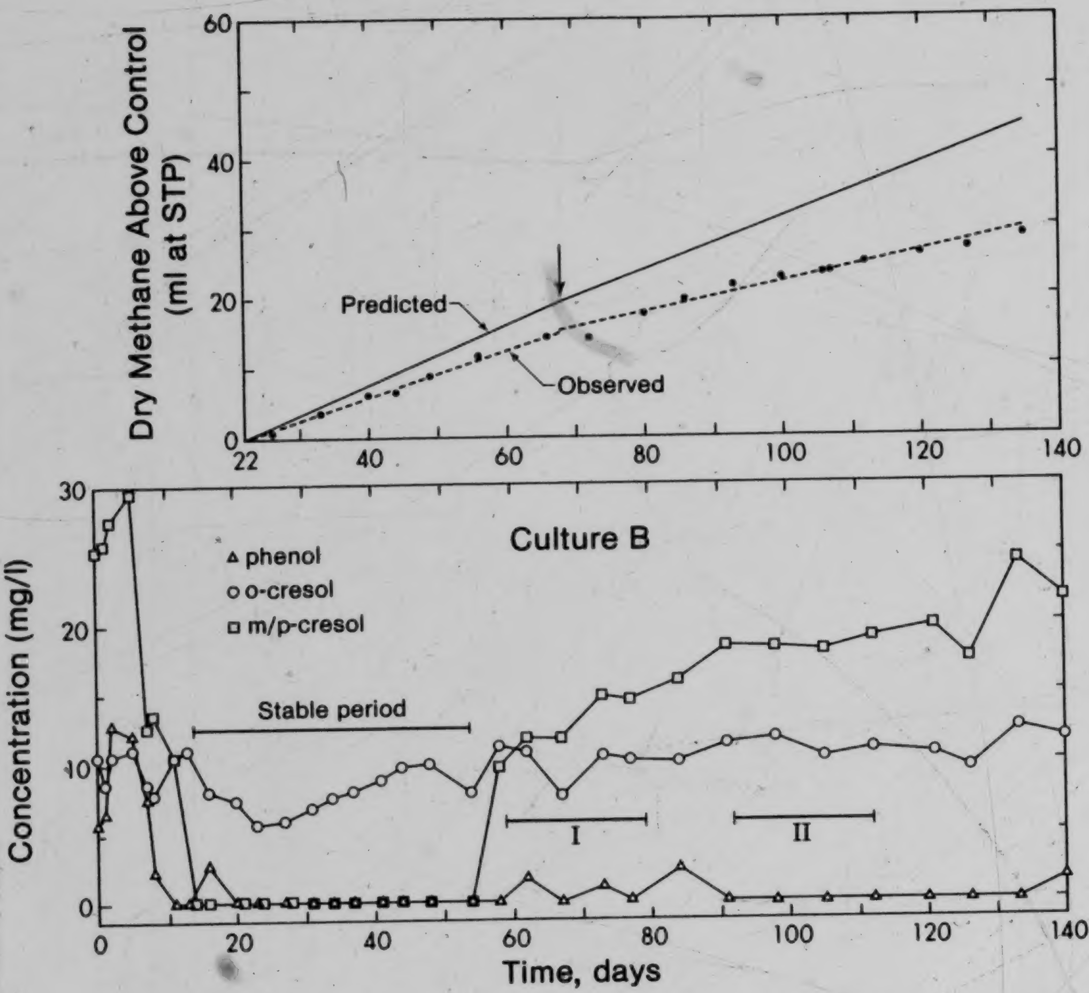


Figure 9.5 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 3 mL of 2% H-coal effluent each day.

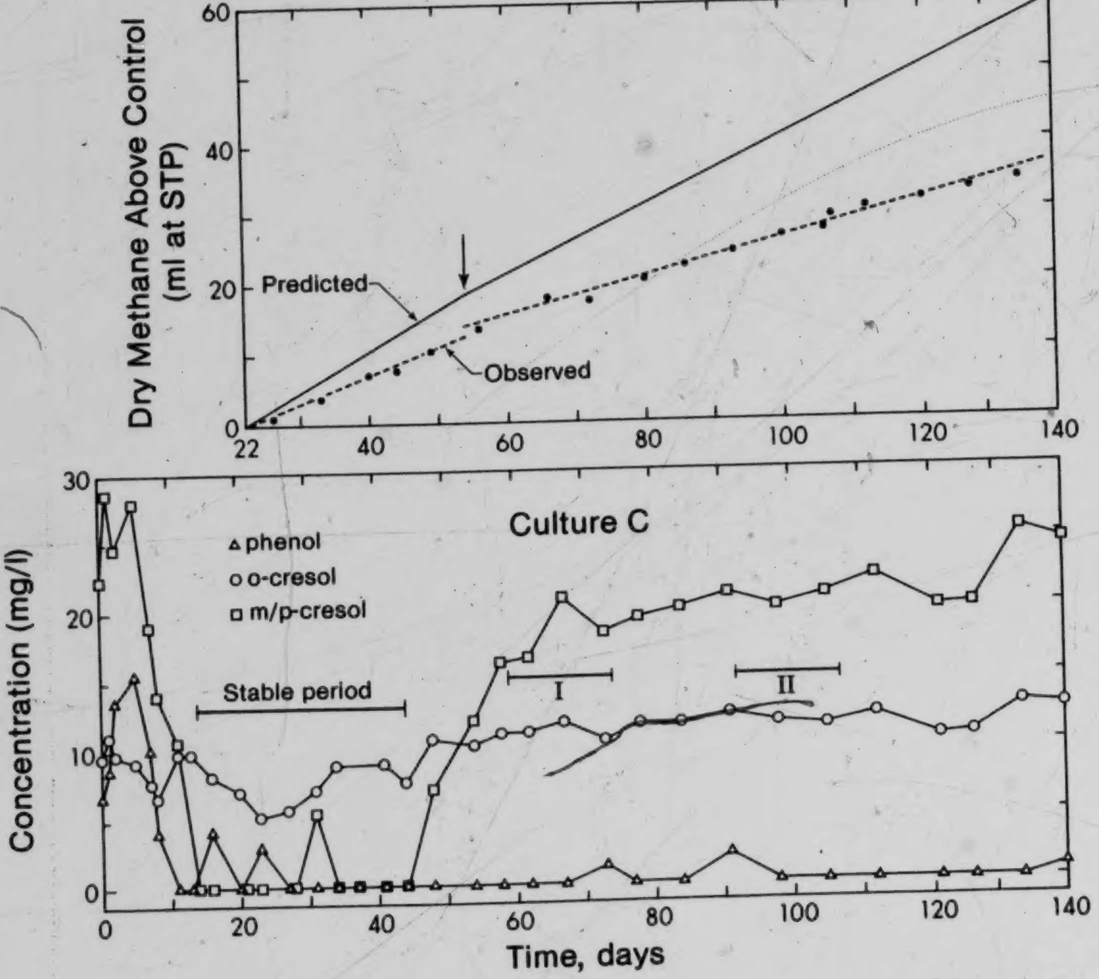


Figure 9.6 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 4 mL of 2% H-coal effluent each day.

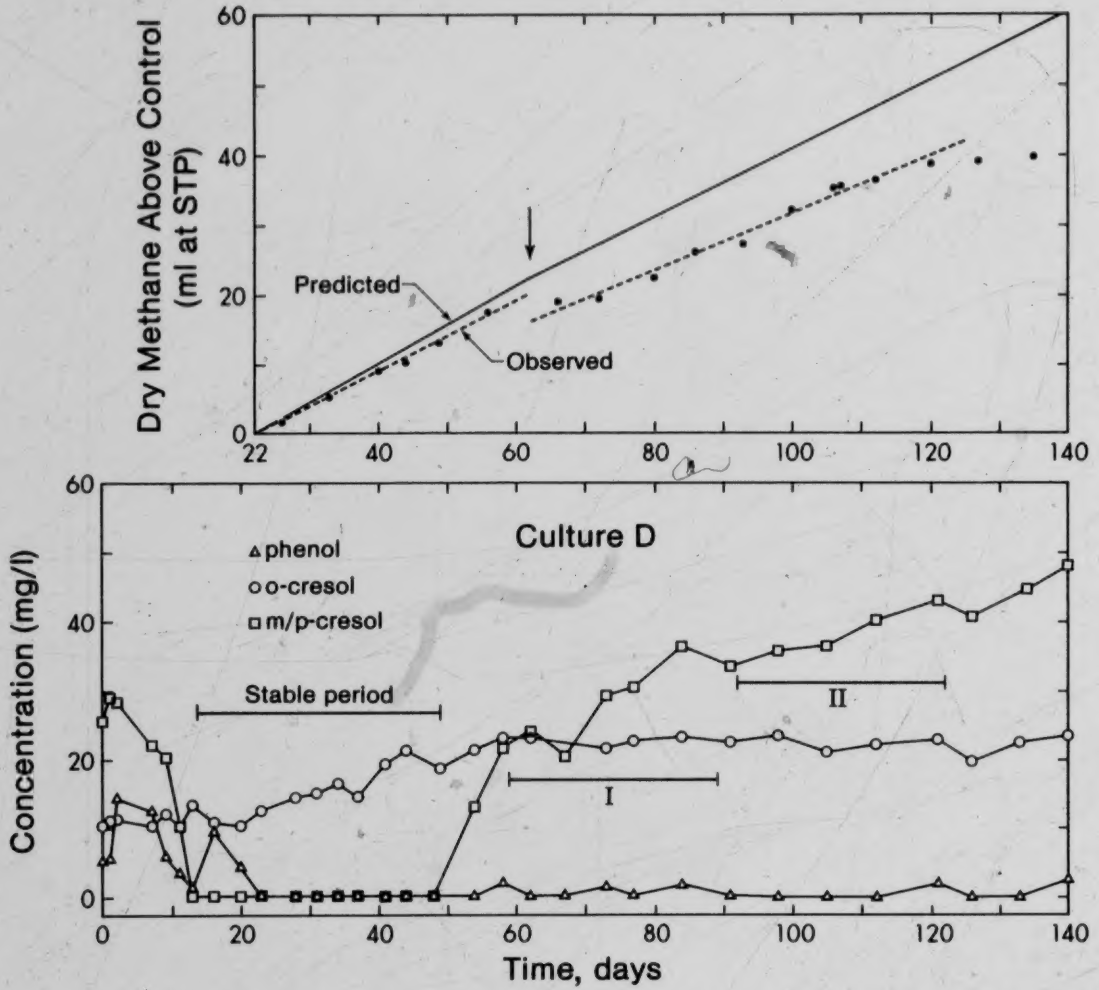


Figure 9.7 **Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 2 mL of 4% H-coal effluent each day.**

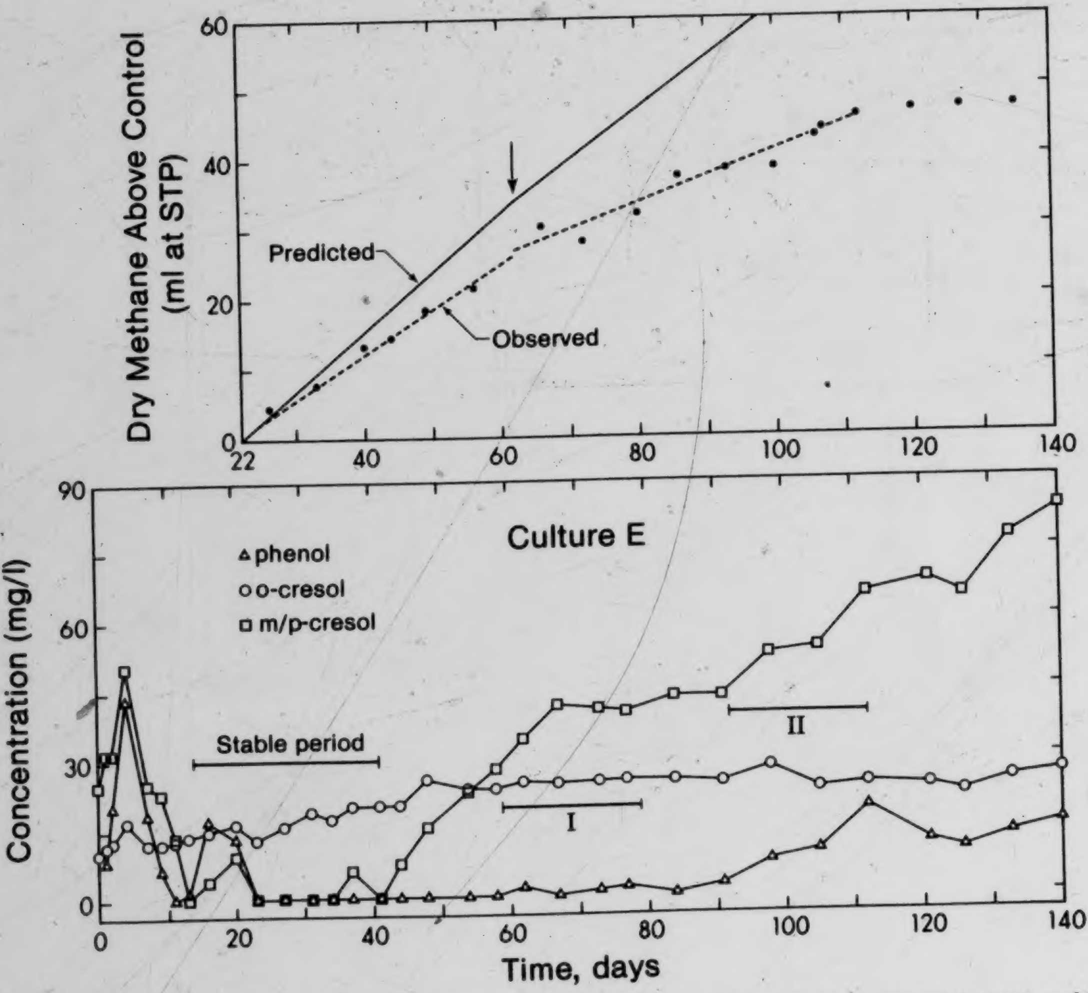


Figure 9.8 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 3 mL of 4% H-coal effluent each day.

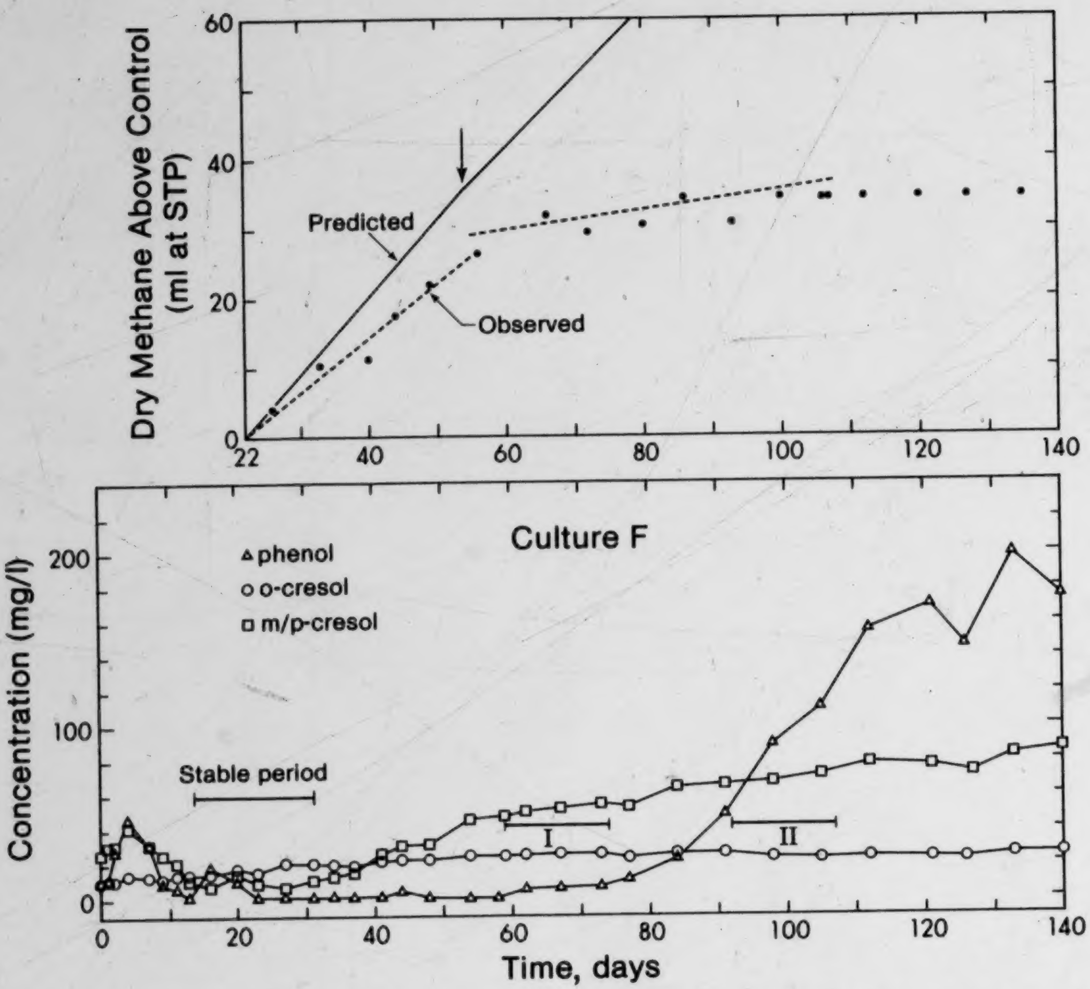


Figure 9.9 Methane production and effluent phenolic concentrations from a 50 mL semicontinuous culture receiving 4 mL of 4% H-coal effluent each day.

it was expected to reach a constant concentration of 11.7 mg/L. In bottles D, E, and F the o-cresol concentration started at near 11 mg/L since these cultures had been enriched on 2% H-coal effluent, and increased to near 24 mg/L after a number of feedings with 4% H-coal feed which contained 23.4 mg/L o-cresol.

All six semicontinuous cultures provided good removal of phenol, m- and p-cresol from the H-coal wastewater for varying lengths of time. The "stable period" began on day 14 as shown in Figures 9.4-9.9. During this period, some spikes of elevated m/p-cresol concentrations were observed (e.g. days 16 and 34, Figure 9.4) and in some cultures, particularly F (Figure 9.9), the m/p-cresol concentrations remained low, but measurable. The stable period was considered to be terminated when two successive increases were observed in the m/p-cresol concentration. The time just prior to these two elevated concentrations marked the end of the stable period. The durations of the periods are summarized in Table 9.2. These data clearly show that an increased loading rate (as expressed by the m/p-cresol loading rate) corresponded to a decreased number of days in the stable period. For example, with a loading rate of 0.066 mg/day (culture A) the stable period lasted 53 days while 0.264 mg/day (culture E) gave a stable period of only 17 days. Cultures C and D received different strength feed solutions but had the same loading rates (Table 9.2). These cultures had nearly the same stable removal period (30 and

Table 9.2 Removal of m/p-cresol from semicontinuous cultures receiving diluted H-coal effluent.

Culture	Draw and feed volume/day (mL)(1)	Feed Solution(2)	m/p-cresol loading rate (mg/day)	Stable removal period (days)
A	2	2%	0.066	53
B	3	2%	0.099	40
C	4	2%	0.132	30
D	2	4%	0.132	34
E	3	4%	0.198	27
F	4	4%	0.264	17

(1) Fed to 50 mL culture
(2) H-coal effluent (V/V)

34 days, respectively).

During the early stages of m/p-cresol accumulation in cultures B to F, composite effluent samples (designated I) were collected from day 59 until a 60 mL aliquot was obtained (Figures 9.5-9.9). Starting on day 92, composite samples designated II, were collected from each of the six cultures. The m- and p-cresol concentrations in each sample are summarized in Table 9.3. These data show that m-cresol was the first to accumulate since p-cresol was found in only one the composite I samples (culture F) while all five cultures contained m-cresol. In composite II, m-cresol - but no p-cresol - was found in cultures A, B and D while increasing concentrations of both m- and p-cresols were found in cultures C, E and F (Table 9.3).

Phenol concentrations also began to increase in the more heavily loaded cultures. In culture F (Figure 9.9), the phenol concentration was near 180 mg/L by day 140. Since the 4% H-coal effluent feed contained 196 mg/L, there was very little phenol degradation occurring in the culture. A smaller increase was observed in culture E (Figure 9.8). Betweeen days 23 to 90, there had been consistently less than 2 mg/L phenol in the effluent from this culture but on day 140, a concentration of 15 mg/L phenol was present.

The phenolic analyses of the effluents from these semicontinuous cultures showed that as the performance of the cultures deteriorated, there was a progressive accumulation of first m-cresol, then p-cresol and finally

Table 9.3 Concentrations of m- and p-cresol in composite effluent samples from semicontinuous cultures receiving diluted H-coal effluent.

	Composite I		Composite II	
Culture	m-cresol (mg/L)	p-cresol (mg/L)	m-cresol (mg/L)	p-cresol (mg/L)
A	NA[1]	NA	13	0
B	13	0	17	0
C	17	0	17	<2
D	24	0	30	0
E	30	0	38	11
F	39	3	42	28

[1] Not analyzed

phenol. This is consistent with the profile on degradability lag times indicated by Figure 7.6.

The accumulation of m/p-cresol in the H-coal effluent-fed cultures was scrutinized more closely to determine whether the m-cresol, and later the p-cresol, degrading capabilities of the microbial consortia had completely stopped or had simply slowed down. The m/p-cresol concentrations observed after the end of the stable period were compared to dynamic response curves corresponding to a step decrease in removal rate. The limiting curve would correspond to a step decrease to a zero removal rate which would be simple washout (Figures 9.10 - 9.15).

The phenolic substrates are very water-soluble, and the culture bottles were vigorously mixed after feeding. Assuming a first order removal rate and approximation of a continuous flow stirred tank reactor, the following equation would describe the dynamic response of the effluent concentration (Schroeder, 1977):

$$C(t) = \frac{C_o}{1+\theta k}\{1-\exp[(-t/\theta(1+\theta k)]\} \qquad (9.1)$$

where C = effluent concentration of a given compound at time, t

C_o = influent concentration of the compound in question

θ = hydraulic retention time

and k = a first order removal rate constant

If the removal of the phenolic in question has stopped, then

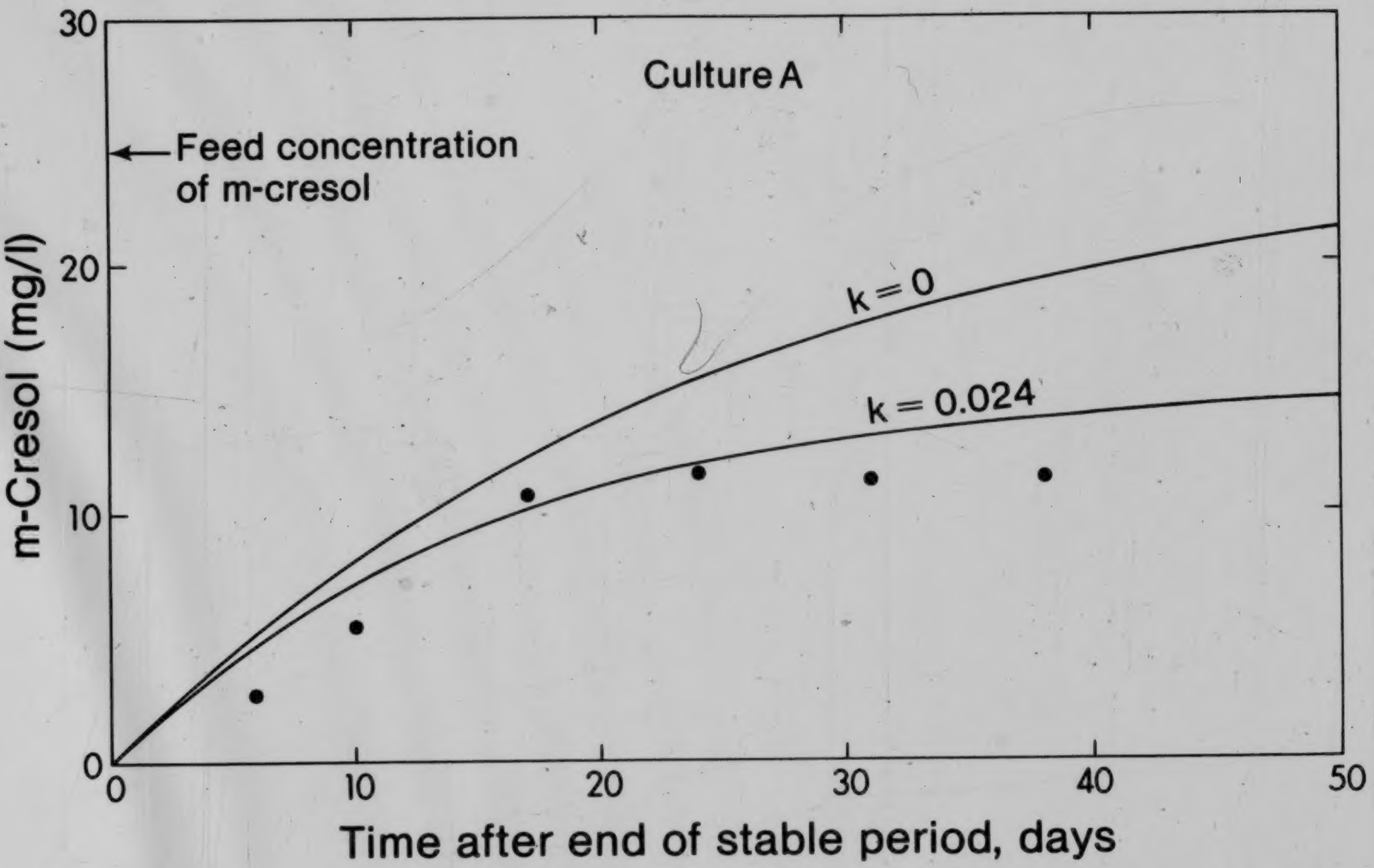


Figure 9.10 Comparison of observed m-cresol concentrations in the effluent from culture A to those expected under washout conditions (k=0).

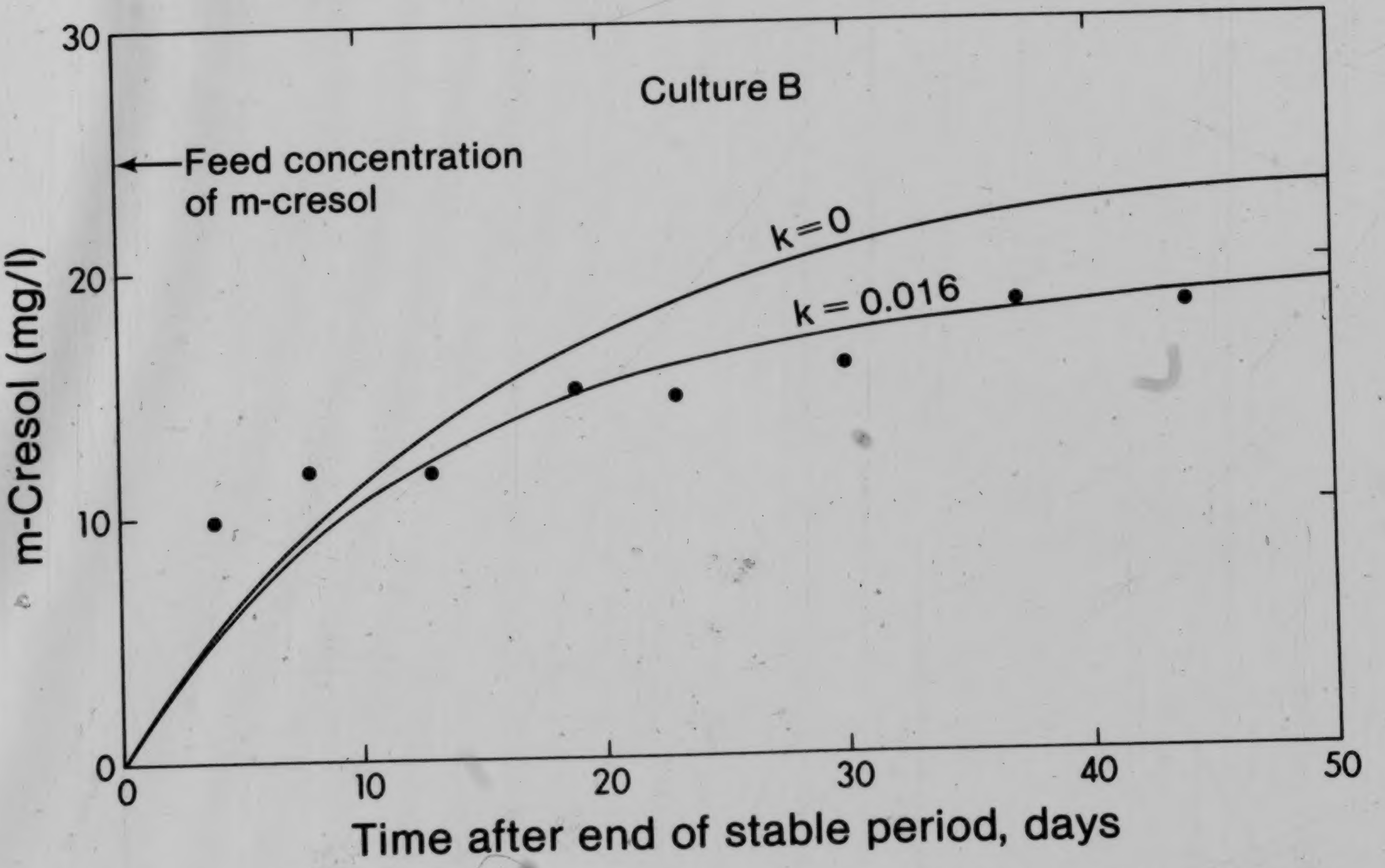


Figure 9.11 Comparison of observed m-cresol concentrations in the effluent from culture B to those expected under washout conditions (k=0).

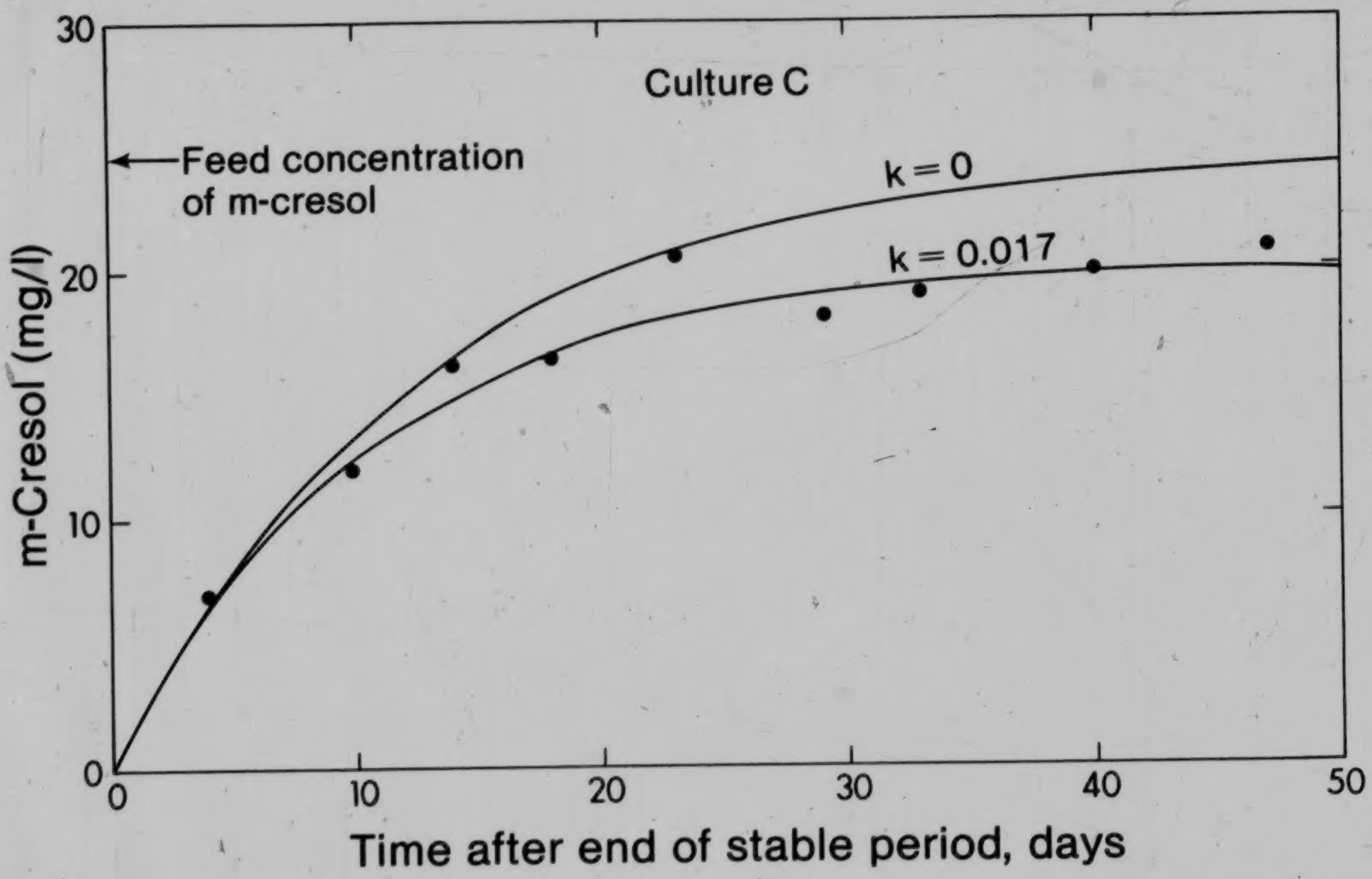


Figure 9.12 Comparison of observed m-cresol concentrations in the effluent from culture C to those expected under washout conditions (k=0).

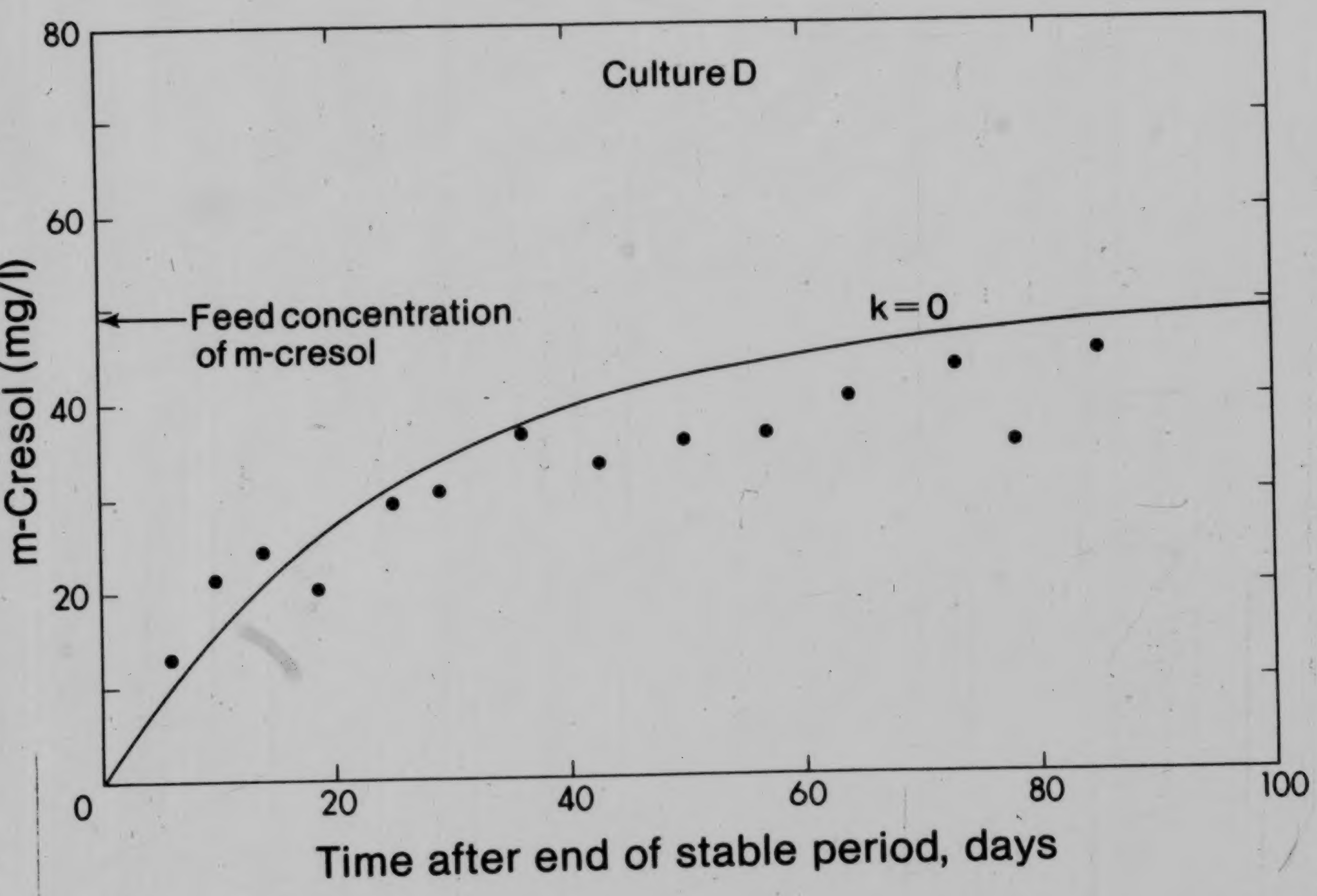


Figure 9.13 Comparison of observed m-cresol concentrations in the effluent from culture D to those expected under washout conditions (k=0).

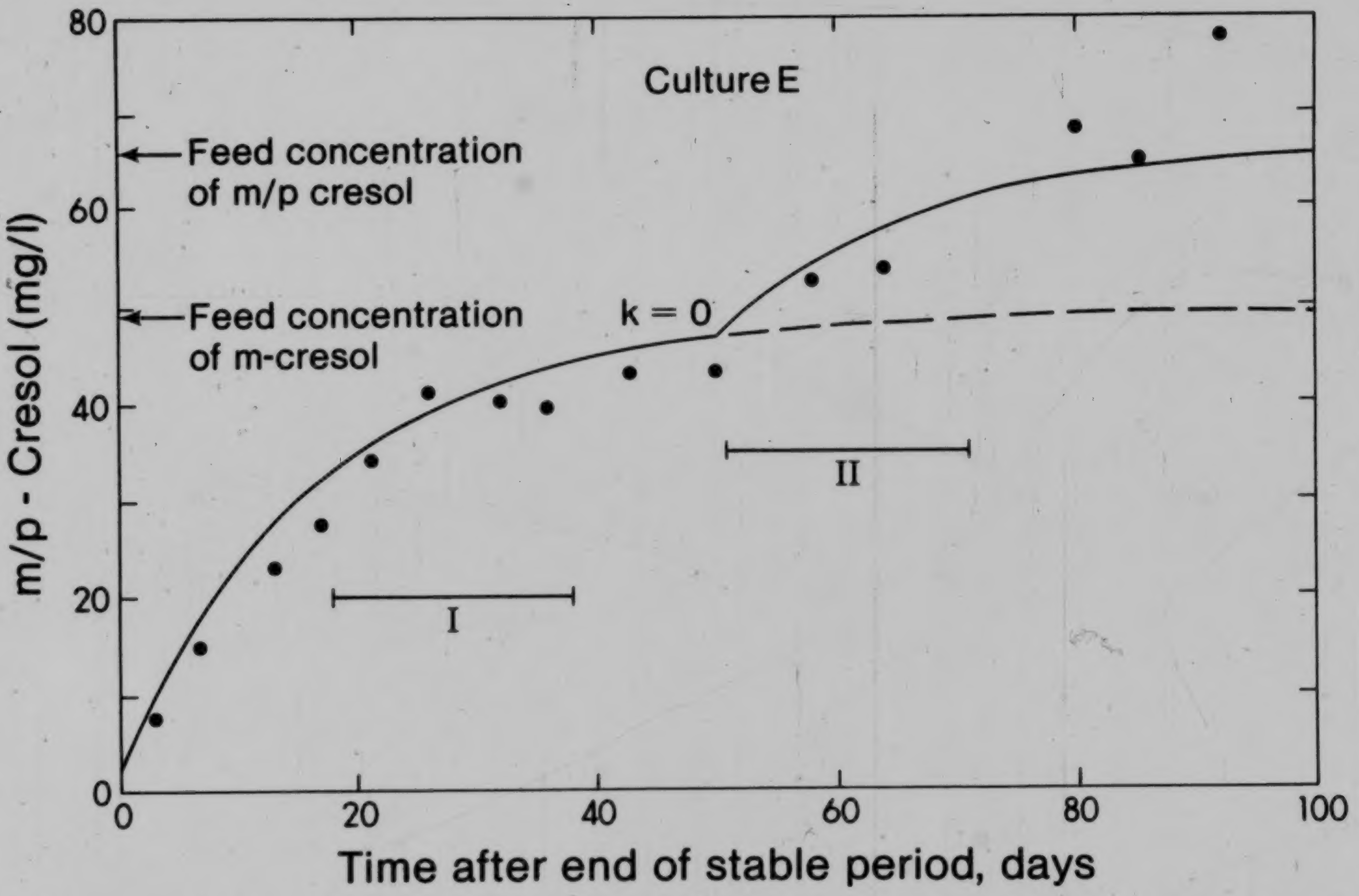


Figure 9.14 Comparison of observed m/p-cresol concentrations in the effluent from culture E to those expected under washout conditions (k=0) for both isomers. From time zero to day 50, only m-cresol washout was calculated. After day 50, the calculated p-cresol washout concentrations were added to those of m-cresol (broken line). I and II are intervals when composite effluent samples were collected.

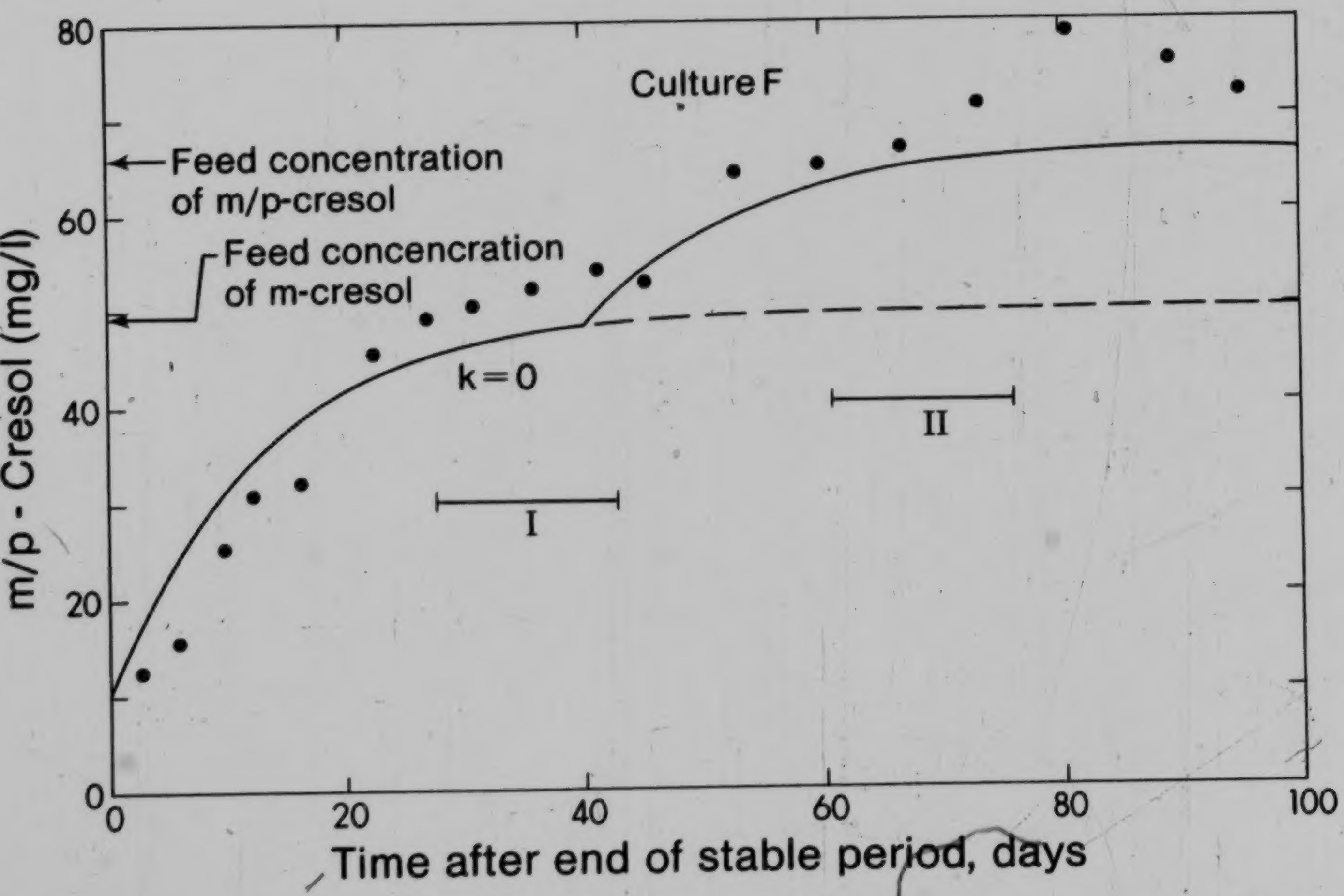


Figure 8.15 Comparison of observed m/p-cresol concentrations in the effluent from culture F to those expected under washout conditions (k=0) for both isomers. From time zero to day 40, only m-cresol washout was calculated. After day 40, the calculated p-cresol washout concentrations were added to those of m-cresol (broken line). I and II are intervals when composite effluent samples were collected.

k=0, and equation (9.1) becomes

$$C(t) = C_0 [1-\exp(-t/\theta)] \qquad (9.2)$$

which predicts the washout concentration as a function of time.

The value of k can be estimated when the system is at steady state and the effluent concentration remains constant. This occurs when t becomes very large and equation (9.1) becomes

$$C = C_0/(1+\theta k) \qquad (9.3)$$

Thus, when C becomes constant at large values of t ($>4\theta$), the system has virtually achieved steady state and k can be calculated using a rearranged form of equation (9.3)

$$k = [(C_0/C) - 1]/\theta \qquad (9.4)$$

Although Figures 9.4 - 9.9 summarize only the data collected during the first 140 days of incubation, all six semicontinuous cultures were maintained for a total of 188 days. This extended incubation time allowed the calculation of mean m-cresol concentrations in the effluent at times greatern than 4θ after the end of the stable period.

Figure 9.10 shows the m-cresol washout curve for culture A (k=0) and the observed m-cresol concentrations over the 50 days following the end of the stable period.

These times correspond to days 67 to 117 in Figure 9.4. Since no p-cresol was detected in composite sample II, only the m-cresol washout curve was considered. (Later figures consider both m- and p-cresol washout.) The observed m-cresol concentrations do not follow the predicted washout curve. They plateau at a mean concentration, C, of 14.9 mg/L after $t = 4\theta$ instead of approaching the feed concentration, C_0, of 24.6 mg/L. Using these two values and $\theta = 25$ days, equation (9.4) gives $k = 0.024$ day^{-1}. The value for k prior to the end of the stable period was also estimated. During this period, the m-cresol concentration was below the detection limits of the GC and therefore in equation (9.4), $C < 1$ mg/L. This gave $k > 0.94$ day^{-1}. The data in Figure 9.10 suggest that at the end of the stable period, the rate of m-cresol removal decreased in culture A. However, removal apparently continued at a lower rate.

Figures 9.11 and 9.12 show similar results for cultures B and C, respectively. The 50-day intervals correspond to days 54 to 104 in Figure 9.5 (culture B) and days 44 to 94 in Figure 9.6 (culture C). Composite effluent samples from culture B showed no p-cresol accumulation during this time period (Table 9.3) and only trace amounts of p-cresol were found in composite II from culture C. Therefore, only the m-cresol washout was considered. As with culture A, the observed m-cresol concentrations in cultures B and C tended to plateau at levels below those predicted for washout conditions (Figure 9.11 and 9.12, respectively). These observations indicate that the removal rate k was greater than zero. Table 9.4 summarizes the estimated k values observed both during and after the stable periods for each of the six semicontinuous cultures.

During the stable periods, there was <1 mg/L m-cresol detected in the effluents from cultures B and C. Thus the estimated k values were >1.41 day^{-1} and >1.89 day^{-1}, respectively. The k value estimated for culture C is greater than that for culture B because θ was only 12.5 days for C versus 16.7 days for B. The other values used in equation (9.4) were the same for these two cultures. After the stable period, both cultures showed essentially the same removal rate constant ($k = 0.016$ day^{-1} for culture B and $k = 0.017$ day^{-1} for culture C). These data suggest that at the end of the stable period, the m-cresol removal capabilities of these cultures decreased drastically but did not completely cease. This type of response, wherein some apparent removal continues, was observed in all three cultures which received 2% H-coal feed solutions.

The responses of the cultures D, E and F which received 4% H-coal feed were quite different from those of the 2%-fed cultures. As summarized in Table 9.4, the removal rate k was essentially zero after the end of the stable period. That is, the observed m-cresol concentrations very nearly followed the expected concentrations for washout conditions (Figures 9.13 to 9.15).

Table 9.4 Estimated first order rate constants, k, for m-cresol removal in semicontinuous cultures receiving diluted H-coal effluent(1).

Culture	k, during stable period (day^{-1})	k, after stable period (day^{-1})
A	>0.94(2)	0.024
B	>1.41(2)	0.016
C	>1.89(2)	0.017
D	>1.93(2)	~ 0(3)
E	1.32	~ 0(3)
F	0.31	~ 0(3)

(1) Equation (9.4) used to calculate k.

(2) Mean effluent concentration of m-cresol <1 mg/L.

(3) Experimental data were very near values expected for washout conditions.

The results for culture D are shown in Figure 9.13. During this 92-day period (corresponding to days 48 to 140 in Figure 9.7), the m-cresol concentration continuously increased following a washout curve corresponding to negligible removal rate (k=0). These data, along with results in Table 9.3, suggest that at the end of the stable period, the removal of m-cresol stopped but p-cresol removal continued over the remainder of the study period.

Analyses of the effluent from cultures E and F during the stable period frequently showed the presence of m/p-cresol. Since the data in Table 9.3 shows little or no p-cresol in the composite I samples from these two cultures, it is very likely that only m-cresol was in the effluents during their stable periods. The mean m-cresol concentrations during this time were 2.1 mg/L and 10.1 mg/L for cultures E and F, respectively. Thus, the rate constants for m-cresol removal during the stable periods were 1.32 day^{-1} and 0.31 day^{-1}, respectively (Table 9.4).

The washout curves for cultures E and F are shown in Figures 9.14 and 9.15, respectively. These show the expected m/p-cresol concentrations if at first, only m-cresol was in the effluent and then later, if both m- and p-cresol were in the effluent. It was presumed that p-cresol did not accumulate appreciably until after the m-cresol concentration was near the level of the feed concentration (49.2 mg/L). Neither curve starts at the origin since the effluent m-cresol concentrations at the end of the stable period were not zero. The mean concentrations of 2.1 mg/L for culture E and 10.1 mg/L for culture F were used as estimates of the effluent m-cresol concentrations at the end of the stable period (time zero, Figures 9.14 and 9.15). In order to calculate the washout curves which accounted for this "background" m-cresol concentration in the effluent, C_0' was calculated as the difference between the feed m-cresol concentration (49.2 mg/L) and the background m-cresol concentration. Thus C_0' was 47.1 mg/L for culture E and 39.1 mg/L for culture F. These C_0' values were used in place of C_0 in equation (9.2) to give the appropriate value at time t on the washout curve (k=0). Each of the m-cresol curves have been shown for a 100-day period (Figures 9.14 and 9.15). These intervals correspond to days 41 to 140 for culture E (Figure 9.8) and days 31 to 131 for culture F (Figure 9.9).

The observed m/p-cresol concentrations in Figures 9.14 and 9.15 indicated many were above the m-cresol feed concentration. Thus washout curves for p-cresol were calculated (based on a feed concentration of 16.8 mg/L) and the p-cresol washout concentrations were added superimposed on those for m-cresol. Inspection of the data from culture E suggested that the addition of these two cresol washout concentrations should start at day 50 (Figure 9.14). Similarly, in the case of culture F, the addition should start on day 40 (Figure 9.15). The validity of these choices is supported by the data from the analyses of composites I and II given in Table 9.3. In the case of culture E (Figure 9.14), no p-cresol was observed in composite I but it was present in composite II. For culture F (Figure 9.15), a low concentration of p-cresol was found in composite I indicating that this isomer was beginning to accumulate within 40 days after the end of the stable period.

The observed m- and p-cresol concentrations closely follow the washout curves for cultures E and F in Figures 9.14 and 9.15, respectively. These results suggest that the m-cresol removal activity essentially stopped at the end of the stable period in the cultures which received 4% H-coal feed. These observations agree with the findings in culture D. During the period of m-cresol accumulation in the cultures E and F, p-cresol removal appears to have been uninhibited. However, in both cases, when the m-cresol concentrations reached near the feed concentration, the p-cresol removal seems to have stopped abruptly and the effluent concentrations of m/p-cresol followed the washout curves assuming cessation of p-cresol removal.

The changes in the microbial activities which caused altered effluent concentrations were also reflected in the rates of methane production. The top panels in Figures 9.4-9.9 compare the predicted and observed absolute amounts of methane in each culture. Methane measurements were started on day 22 when the headspace gas in each culture bottle was exchanged to remove all accumulated methane. At that time the major phenolic in the effluent was o-cresol.

The predictions of the amount of methane production were based on the results of the ultimate methane per unit volume of H-coal effluent fermented in batch cultures (section 8.2.5). For each semicontinuous culture, the prediction was based on two different methane production rates to reflect the change in the microbial activity. During the early stages of methane measurements, phenol, m- and p-cresol were all being removed and the expected rate of methane production was 7.1 mL/mL H-coal effluent. After m-cresol removal had decreased substantially or stopped, the expected rate was 6.1 mL methane/mL H-coal effluent. These two slopes are shown in Figure 9.4-9.9 with arrows indicating when the change of rate appeared to occur based on the slopes of the observed data.

The observed methane values plotted in Figures 9.4-9.9 have been corrected for methane evolution which was not a result of the H-coal effluent in the feed. The two control cultures produced similar amounts of methane and therefore at each measurement time, the average of the two values was calculated and this was subtracted from the methane amounts found in each of the six cultures. Since the expected rate of methane production in culture A was only 0.214 mL/day and the mean methane production rate in the controls was approximately 0.05 mL/day, the control values could not be neglected as was the case in the semicontinuous cultures which received either phenol or p-cresol (section 9.2.1).

Least squares analyses were done to determine the observed rates of methane production and the resulting lines are shown in Figures 9.4-9.9. The data in Table 9.5 summarize the observed rates, the predicted rates and expresses the observed as a percentage of predicted values. In all cases the methane production rates were significantly less than the expected rates (P>0.05).

During the first stage of methane monitoring when the three fermentable phenolics were being degraded, the observed methane formation rates were between 67% and 89% of the predicted values (Table 9.5). The loading rates did not greatly affect the efficiency of methane production during the initial monitoring period. Cultures C and D received the same loading rates (i.e. 0.596 mg/day phenolics) but the hydraulic retention times differed. With the shorter retention time of 12.5 days, culture C produced 0.390 mL methane/day while culture D, which had a 25-day retention time, produced 0.507 mL methane/day. The difference between the two methane production rates was likely due to the higher rate of daily effluent withdrawal from culture C which still contained fermentable substrates such as VOAs.

In general, during the second time interval over which methane production was monitored (Table 9.5), the efficiencies of methane production in the semicontinuous cultures decreased. The notable exception was culture A which produced methane at 75% of the predicted rate during both intervals. The methane production efficiencies of cultures B

Table 9.5 Summary of methane production rates in semicontinuous cultures receiving diluted H-coal effluent.

Culture	Phenolic feed rate[1] (mg/day)	Interval[2] (days)	Rate (mL methane/day)		Efficiency: $\frac{\text{Observed}}{\text{Predicted}} \times 100$
			Observed	Predicted	
A	0.298	22-84	0.214	0.284	75%
		85-135	0.182	0.244	75%
B	0.447	22-68	0.332	0.425	78%
		69-135	0.214	0.365	59%
C	0.596	22-54	0.390	0.568	69%
		55-135	0.268	0.488	55%
D	0.596	22-62	0.507	0.568	89%
		63-120[3]	0.398	0.488	81%
E	0.894	22-62	0.634	0.852	74%
		63-112[3]	0.361	0.732	49%
F	1.19	22-54	0.764	1.14	67%
		55-84[3]	0.125	0.976	18%

[1] Based on total phenolic concentration in Table 8.2.
[2] Refers to Figures 9.4 - 9.9.
[3] Methane production essentially stopped at this time.

and C (which received 2% H-coal effluent in the feed) dropped from 78% to 59% and from 69% to 55%, respectively. Methane evolution remained constant over the remainder of the time period shown in Figures 9.4-9.6.

In contrast, the methane formation essentially stopped during the later stages of the semicontinuous cultures which received 4% H-coal effluent. The higher the feed rate, the shorter the time period for methane formation. Culture D produced methane at 81% of the predicted rate during the second interval until day 120 after which no further methane accummulated. There was also a corresponding increase in m/p-cresol in the culture effluent after day 120 although the phenol concentrations remained low (Figure 9.7). A marked drop in efficiency was observed in culture E which produced methane at only 49% of the predicted rate. By day 112, methane formation stopped and m/p-cresol concentrations began to increase in the effluent from culture E and the phenol concentration remained near 15 mg/L (Figure 9.8). The greatest process upset occurred in culture F which was receiving the highest loading rate. Methane evolution was only 18% of the expected rate during the second interval (Figure 9.9). A sharp increase in phenol concentration in the effluent was also noted after day 84.

During this experiment, it became apparent that the cultures were being overloaded. However, no attempt was made to reduce the loading rate to allow the cultures to degrade the accumulated phenolics. Instead, the daily draw and feed procedures were continued so the responses of and effects on each culture could be monitored. Using this approach it became clear that the appearance of m-cresol in the effluent was an early warning that the treatment process was beginning to fail.

An understanding of the microbiology and biochemistry of m-cresol degradation may provide the key to establishing loading rates and operating conditions for a process to treat phenolic wastewaters. This isomer required the longest acclimation time prior to the start of its degradation and was the first to accumulate in the H-coal-fed semicontinuous cultures.

Anaerobic digestion has become a wide spread and reliable method of treating domestic sewage sludge. This success has, in part, stemmed from the recognization of the fastidious nature of the methanogens and adjusting digester operating parameters (e.g. temperature, pH, loading rate, etc.) to suit these organisms. Since the m-cresol-degrading organisms are even more fastidious than the methanogens, conditions which will maintain their stable activity should establish appropriate operating parameters for the effective treatment of phenolic-wastewaters.

10. SUMMARY AND CONCLUSIONS

The experimental work in this thesis approached aspects of anaerobic treatment of phenolic wastewaters from two different directions. In the majority of the experiments, individual phenolics or synthetic mixtures of phenolics were added to cultures to determine the responses of the methanogenic consortia to these compounds. In the second group of experiments, the treatability of the phenolics in two industrial wastewaters were examined. Results from the work with pure phenolics lead to accurate predictions of the responses of cultures to real wastewaters and observations from the wastewater-fed cultures verified trends suggested by pure substrate work. Studies with H-coal effluent showed that m-cresol was fermentable - an observation which was missed during the initial 45 day screening studies with cultures given pure substrates.

Phenol and a total of 12 alkyl phenolics were tested for fermentability under batch culture conditions. Of these, only phenol, p-cresol and m-cresol were found to be degraded and converted to methane. The non-fermentable dimethylphenols (all six isomers) were not inhibitory to the anaerobic processes until their concentrations exceeded 300 mg/L. Inhibition by the non-fermentable o-cresol was not observed until its concentration exceeded 400 mg/L. When tested individually at 200 mg/L, p-ethylphenol and two isomers of dihydroxytoluene were not inhibitory to the anaerobic process.

Three different anaerobic sludges that had been previously exposed to phenolic compounds were tested to determine whether they contained microbial populations with the ability to degrade a wider range of alkyl phenolics. None of these sludges were more active than domestic anaerobic sewage sludge with respect to the types of alkyl phenolics which they could ferment.

Since the fermentability of m-cresol was only observed late in the study, less information on its degradation and its effects on the cultures is available. Most studies dealt with phenol and p-cresol fermentation. Experiments with these two compounds showed that the phenolic-degrading acid-formers were most sensitive to the toxic effects of the phenolic substrate. As the concentrations of either substrate increased, degradation of the phenolic stopped, but the methanogenic fermentation of non-phenolic substrates continued for phenol concentrations up to 1 200 mg/L or p-cresol concentrations up to 600 mg/L. The methane bacteria, which are usually considered to be most sensitive to environmental factors in an anaerobic digester, were found to be far less sensitive to the phenolic concentrations than were the phenolic-degrading acid-formers.

Within the range of fermentable concentrations, the acclimation times for phenol and p-cresol were found to increase as the concentration of phenolic substrate was increased. This was found both in batch studies with pure phenolic substrates and with H-coal effluent and reconstituted H-coal effluent.

The rates of phenol degradation in batch cultures were studied to determine if they followed the Monod kinetics model which predicts sigmoidal substrate depletion curves. Such curves were not observed and the Monod model was judged to be inappropriate to describe the rate of phenol utilization under these culture conditions. During the period of active phenol degradation in these batch cultures, the rate of substrate disappearance continuously increased until phenol was depleted from the medium. At initial phenol concentrations between 43 and 199 mg/L, the final rates of phenol removal followed first order reaction kinetics.

The role of molecular hydrogen was assessed in batch cultures to determine if the initial rate of phenol degradation could be increased by supplying H_2. Propionate an indirect source of H_2, was added to batch cultures just prior to the start of phenol degradation. Propionate addition actually decreased the initial degradation rate. This observation suggested that the phenol-degraders may also be able to metabolize propionate and that the latter is the preferred substrate. The addition of H_2 at the time when phenol degradation began did not stimulate nor inhibit phenol degradation. Thus, the role of H_2 in the degradation of phenol remains unclear. However, it does not appear to be the limiting factor at the onset of phenol degradation in batch cultures.

During the early screening experiments, some domestic sludge samples would degrade p-cresol at 400 mg/L while others would not. These variations suggested that the phenolic-degraders were numerically only a minor portion of the population. Later analyses using the MPN technique gave low numbers of PDU. However, the applicability of the test was questioned since much larger numbers of phenol degraders had to be present to account for the phenol transport rate required to match the rate of phenol degradation in batch cultures. The likelihood of interspecies involvement in phenol degradation also shed doubt on the use of standard MPN tables which are based upon the assumption of a single active species.

In synthetic mixtures, fermentable phenolics were found to be degraded if the total concentration of phenolics was near or less than 700 mg/L. This was first observed with mixtures containing phenol, o- and p-cresol, and three isomers of dimethylphenol. Later, batch cultures which received up to 10% (V/V) reconstituted H-coal effluent (i.e. 746 mg/L total phenolics) yielded methane from phenol and m/p-cresol. However, 12% reconstituted H-coal effluent (895 mg/L total phenolics) did not produce enhanced methane production. Experiments with synthetic mixtures of m-cresol and fermentable phenolics in batch cultures showed that the presence of the latter compounds reduced the acclimation time for m-cresol in comparison with cultures which contained only m-cresol.

The fermentability of two phenolic wastewaters was assessed. In both cases, the three fermentable compounds (phenol, m- and p-cresol) accounted for >85% of the phenolics which could be monitored by the two GC methods. The coke effluent contained only 381 mg/L total phenolics but it was not readily treated in methanogenic batch cultures. Tests with an ether-extracted effluent indicated the presence of a non-extractable inhibitory species - possibly cyanide.

The H-coal effluent contained an extremely high concentration of phenolics (7 456 mg/L) and required extensive dilution before it could be biologically treated by anaerobic methods. Inhibition caused by this wastewater in excess of that caused by the identifiable phenolics was not caused by the components that remained in the aqueous phase after ether extraction. The inhibition was attributed to some ether-extractable organic compound(s).

The ultimate amounts of methane and gas production from phenol and p-cresol were found to be less than predicted by Buswell's equation (which does not account for the conversion of substrate carbon to cell mass). The expected methane production rate (per mg phenolic substrate) was used to assess the conversions of phenol and p-cresol in individual semicontinuous (draw and feed) cultures. These cultures remained active for long periods of time. The phenol-fed culture produced methane at 86% of the expected rate while the p-cresol-fed culture produced methane at the expected rate.

Semicontinuous cultures were maintained on H-coal effluent at various feed rates and hydraulic retention times. After the initial start-up in each of the six cultures, there were stable periods during which essentially all of the phenol, m-cresol and p-cresol were removed. Lower mass loading rates gave longer stable periods. In every case, m-cresol began to appear in the reactor effluents in increasing concentrations, suggesting dramatic reduction or cessation of m-cresol removal. In the more heavily loaded cultures, p-cresol and finally phenol accumulated. Methane production rates reflected the changes in phenolic substrate removal by these cultures.

Analysis of the dynamic response of the effluent m/p-cresol concentration for these reactors was performed using the assumption that observed values resulted from a change in first order removal rate in a complete mix reactor. Setting this removal rate to zero provided the theoretical washout curves for each reactor. As well, where the ultimate steady state effluent concentration for m-cresol and p-cresol was below the washout concentration the apparent first order removal rates were estimated. The value of this removal rate allowed calculation of an expected dynamic response following onset of the lower removal rate.

The dynamic response analyses showed that the three cultures which received 2% H-coal effluent feed maintained their p-cresol removal rate throughout the study period. However, the end of the stable period was marked by a substantial reduction in the m-cresol removal rate resulting in a consistent rise in m-cresol occurring in the reactor effluent.

In contrast, for the three cultures receiving 4% H-coal effluent the removal rate for m-cresol apparently dropped to zero. This resulted in straight washout of m-cresol from the reactors. Forty to fifty days after the end of the stable period, effluent p-cresol began to rise for the two most heavily loaded cultures. The p-cresol dynamic response was also consistent with complete washout (zero removal rate). These results suggest that the microbial population had quickly and completely lost its ability to degrade these two compounds after the stable period under the high loading conditions.

Given the importance of phenolic wastewaters and the promise of anaerobic processes, there was a need to better understand the factors affecting the degradation of phenol and alkyl phenolics by methanogenic consortia. Furthermore, the effects of these compounds on the microbial population must be understood. The results obtained provide important insight in these areas. Specifically they show which phenolics can be removed and which will remain untreated. These data indicate that low initial phenolic concentrations in a reactor will provide faster acclimation and start-up and they suggest that m-cresol may be the "indicator" compound to monitor the performance of the treatment process. Finally, data has been obtained to predict methane generation from phenolic substrates which will allow assessment of pilot scale treatment performance.

The anaerobic fermentation is not capable of degrading all of the wide range of phenolics found in a wastewater, but the process could be used as a first biological treatment step to remove the majority of organic carbon which is commonly present as fermentable phenolics. Final "polishing" could then be done in an aerobic biological system which would readily mineralize the substantially reduced organic loading containing the non-fermentable phenolic compounds.

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